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Increased Expression of the Na,K-ATPase alpha4 Isoform Enhances Sperm Motility in Transgenic Mice[1]

Tamara Jimenez, Gladis Sanchez, Jeffrey P. McDermott, Anh-Nguyet Nguyen, T. Rajendra Kumar, and Gustavo Blanco[2]

Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas

ABSTRACT

The Na,K-ATPase alpha4 (ATP1A4) isoform is specifically expressed in male germ cells and is highly prevalent in spermatozoa. Although selective inhibition of alpha4 activity with ouabain has been shown to affect sperm motility, a more direct analysis of the role of this isoform in sperm movement has not yet been demonstrated. To establish this, we engineered transgenic mice that express the rat alpha4 isoform fused to green fluorescent protein in male germ cells, under the control of the mouse protamine 1 promoter. We showed that the rat *Atp1a4* transgene is expressed in mouse spermatozoa and that it is localized to the sperm flagellum. In agreement with increased expression of the alpha4 isoform, sperm from transgenic mice displayed higher alpha4-specific Na,K-ATPase activity and binding of fluorescently labeled ouabain than wild-type mice. In contrast, expression and activity of ATP1A1 (alpha1), the other Na,K-ATPase alpha isoform present in sperm, remained unchanged. Similar to wild-type mice, mice expressing the alpha4 transgene exhibited normal testis and sperm morphology and no differences in fertility. However, compared to wild-type mice, sperm from transgenic mice displayed plasma membrane hyperpolarization and higher total and progressive motility. Other parameters of motility also increased, including straight-line, curvilinear, and average path velocities and amplitude of lateral head displacement. In addition, sperm from the transgenic mice showed enhanced sperm hyperactive motility, but no changes in progesterone-induced acrosome reaction. Altogether, these results provide new genetic evidence for the role of the ATP1A4 isoform in sperm motility, under both noncapacitating and capacitating conditions.

gamete biology, Na,K-ATPase, sperm, sperm motility and transport

INTRODUCTION

The exchange of cytoplasmic Na^+ for extracellular K^+ catalyzed by Na,K-ATPase plays a central role in the physiology of most cells [1]. The Na^+ and K^+ gradients generated by the Na,K-ATPase are essential in maintaining cell ion homeostasis, cell resting membrane potential, and the secondary transport of other ions, solutes, and water across the cell surface [2–4]. Na,K-ATPase is an oligomer composed of two major polypeptides, the α and β subunits [5, 6]. The α or catalytic subunit of Na,K-ATPase directly participates in the ion translocation and hydrolytic activity of the enzyme, and in the binding of ouabain, a potent inhibitor of Na,K-ATPase [1, 7].

Four structural variants or isoforms of the Na,K-ATPase α subunit, termed α1, α2, α3, and α4, are expressed in mammalian tissues [8, 9]. Each of these α polypeptides has a particular cell-specific pattern of expression and peculiar enzymatic properties [8, 10]. Whereas α1 is found in nearly every tissue, the other α polypeptides are more restricted in their expression and appear to perform cell-specific tasks [9, 11]. Spermatozoa express the ubiquitous α1 isoform and a unique α isoform, the α4 polypeptide [12–16]. In these cells, the α4 isoform is the predominant catalytic subunit of the Na,K-ATPase and its activity is responsible for approximately two thirds of the total Na^+ and K^+ active transport of the male gamete [17]. We have previously shown that α4 exhibits distinct kinetic properties, including a peculiar high sensitivity to ouabain [18, 19]. Interestingly, treatment of spermatozoa with ouabain concentrations that selectively inhibit the Na,K-ATPase α4 isoform decreases sperm motility [16, 18], and affects membrane potential and the ability of the cells to maintain normal intracellular pH and calcium levels [16, 18, 20].

Although important progress has been made in understanding the function of the Na,K-ATPase α4 isoform, through its inhibition with ouabain in vitro [16–18, 21], a more direct approach to understanding the function of this polypeptide in vivo is required. This is an important step in deciphering the biological relevance of α4 in male fertility. In the present work, we have investigated the function of α4 in spermatozoa by transgenic expression of the rat *Atp1a4*, fused to green fluorescent protein (GFP), in mice. To achieve this, we generated transgenic mice with specific expression of α4 in male germ cells using the full-length protamine 1 promoter from mouse. Our results show that expression of rat α4 in sperm from transgenic mice resulted in increased Na,K-ATPase α4 activity and ouabain binding, decreased plasma membrane potential, and enhanced values for multiple parameters of sperm motility, including hyperactive motility. In contrast, higher expression of α4 did not affect the development of the acrosome reaction in the male gamete. These results further demonstrate the biological relevance of α4 and genetically demonstrate that expression and activity of this isoform is directly correlated with sperm motility.

MATERIALS AND METHODS

Generation of Transgenic Animals

A cDNA construct was made that contained the coding sequence of the rat Na+/K+-transporting ATPase, α4 polypeptide fused to GFP at the 3′ end of the cDNA. The GFP was included to provide a reporter for monitoring the expression of the *Atp1a4* transgene. To make this construct, the *Atp1a4* cDNA,

[1]Supported by National Institutes of Health grants HD043044 and HD055763.
[2]Correspondence: Gustavo Blanco, Department of Molecular and Integrative Physiology, 3901 Rainbow Boulevard, Kansas City, KS 66160. FAX: 913 588 7430; e-mail: gblanco@kumc.edu

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in which the stop codon had been deleted, was subcloned in front of the GFP sequence as a *Hin*dIII and *Bam*HI fragment in pEGFP vector (Clontech). No additional DNA sequence between *Atp1a4* and GFP was introduced. This construct was then transferred to the expression vector, pPrCExV1, downstream of the mouse protamine 1 promoter, using *Hin*dIII and *Xba*I sites at the 5′ and 3′ ends, respectively. The pPrCExV1 vector was a generous gift of Robert Braun (The Jackson Laboratory). The cDNA containing the *Atp1a4*-GFP construct was linearized with PstI and microinjected into the pronucleus of one-cell embryos, and these were then surgically transferred into pseudopregnant recipient C57BL/6J female mice following standard procedures [22]. To screen for the transgene, DNA was prepared from tail biopsies using the REDExtract-N-Amp Tissue PCR Kit (Sigma Chemical Co.). The presence of the *Atp1a4*-GFP sequence was detected by PCR, using primers specific to the 3′ end of *Atp1a4* (5′-GCTTGCCTGTCCTACATTCC-3′) and to the 5′ end of GFP (5′-GTCCTCCTTGAAGTCGATGC-3′), which amplified a 615-bp product (data not shown). Two positive founders were obtained, from which two identical lines were established. All experimental protocols used in this work were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

Sperm and Testis Preparation

Spermatozoa were obtained from the cauda of adult mice epididymides. After dissection of the epididymis, the caudal portion was separated and was cut at various points with a razor blade to allow collection of spermatozoa as described [23]. For this, the tissue was placed in modified Tyrode medium [24] containing 100 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5.5 mM glucose, 0.8 mM pyruvic acid, 4.8 mM lactic acid, and 20 mM Hepes (pH 7.4), and was incubated for 10 min at 37°C. After this time, spermatozoa were collected from the supernatant, centrifuged at 300 × *g* for 30 sec, and resuspended in modified Tyrode medium. Cells were counted with a hemocytometer and used for the different assays. In some experiments, cells were placed in modified Tyrode medium supplemented with 1.7 mM $CaCl_2$, 25 mM sodium bicarbonate, and 0.5% bovine serum albumin, factors that are known to support sperm capacitation [25]. Depending on the type of assay, cells were used either intact or homogenized using a glass-glass homogenizer.

Testis from wild-type and α4 transgenic mice were used for immunoblot analysis, as will be described below, and for histological analysis. For the morphological studies, testes were dissected and fixed with buffered formalin phosphate (Fisher Scientific). After embedding in paraffin, the tissue was sectioned in 10-μm sections, treated with xylene and ethanol to remove the paraffin, and stained with hematoxylin and eosin.

SDS-PAGE and Immunoblot Analysis

Protein expression was analyzed by SDS-PAGE (7.5% gel) and immunoblotting on testis samples. Testes from wild-type and transgenic mice were dissected, then homogenized using a glass-Teflon homogenizer, and plasma membrane fractions were prepared as described [19]. Samples were subjected to SDS-PAGE, and proteins were transferred onto nitrocellulose membranes (Nitrobind; Osmonics Inc.) and immunoblotted as previously described [19]. Primary antibodies against the rat Na,K-ATPase α4 isoform and an anti-GFP antibody were used to identify the α4:GFP construct. The α4 isoform was detected with a polyclonal antiserum generated in chicken [18]. For α1, the C464-CB antiserum was used [26]. To identify GFP, a polyclonal antiserum from Sigma Chemical Co. was used. Horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) and chemiluminescence were used for visualizing the antigen-antibody complexes.

Immunocytochemistry

Immunocytochemistry was performed on caudal epididymal spermatozoa, which were plated on 11-mm glass coverslips in 24-well tissue culture plates. Cells were fixed in 4% paraformaldehyde (buffered formalin phosphate; Fisher Scientific) and samples were then processed for immunocytochemistry as described [18]. Briefly, spermatozoa were permeabilized with 0.3% Triton X100 in 25 mM Hepes, pH 7.4, 150 mM NaCl, and 1 mM ethylene glycol-bis (2 aminoethyl-ether)-N, N, N′, N′-tetraacetic acid (EGTA; HBS). After blocking for 2 h at room temperature with 0.2% bovine serum albumin and 2% normal goat serum in HBS, the primary antibodies against the Na,K-ATPase α4 isoform and anti-GFP were applied. The α4 isoform displays a high overall degree of homology among species, with approximately 93% identity between the mouse and rat polypeptides. However, the N-terminal portion of α4 is one of the few regions in the polypeptide that is not conserved and only partially shared between mouse and rat, providing an opportunity for immunological distinction of the isoform between the two species. Thus, we detected the α4 transgene with a polyclonal antiserum that we generated in rabbit against the N-terminal portion (KEKEVEAPGELNQKPRPSTR) of the rat α4 isoform. For GFP, the anti-GFP antiserum mentioned above was used. Following overnight incubation with the primary antibodies at 4°C, samples were washed 3 × 15 min each and treated with secondary antisera conjugated to Alexa Fluor 488 (Molecular Probes). After washing as mentioned above, samples were mounted on slides using SlowFade mounting solution (Molecular Probes) containing 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) to stain the cell nuclei. Fluorescent digital images were obtained using a Nikon Eclipse E600 microscope.

Na,K-ATPase Assays

Na,K-ATPase activity was assayed on sperm homogenates from wild-type and rat α4-expressing mice through determination of the initial rate of release of $^{32}P_i$ from γ[^{32}P]-ATP as previously described [27]. The ATPase activity of 10 μg of total protein per sample was measured. Protein assays were performed using the dye-binding assay from Bio-Rad. Na,K-ATPase was assayed 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, 30 mM Tris-HCl (pH 7.4), 3 mM ATP with 0.2 μCi γ[^{32}P]-ATP in the presence and absence of the indicated ouabain concentrations. Samples were incubated for 30 min at 37°C, reaction was stopped, released ^{32}Pi-Pi was converted to phosphomolybdate and extracted with isobutanol, and 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. Specific overall Na,K-ATPase activity was determined as the difference in ATP hydrolysis in the absence and presence of 1 mM ouabain, which is known to completely block the rodent Na,K-ATPase. Activity of the α4 isoform was defined as that sensitive to 10^{-6} M ouabain, a ouabain concentration that we found to selectively block α4 in previous studies of the kinetic properties of the Na,K-ATPase of sperm [19]. Curve fitting of the experimental data was performed using a Marquardt least-squares nonlinear regression computing program (Sigma Plot; Jandel Scientific).

Ouabain Binding Assay

Ouabain binding capacity of spermatozoa was measured in intact cells using the fluorescently labeled form of ouabain, bodipy-ouabain (Invitrogen). Sperm from wild-type and transgenic mice was placed in modified Tyrode medium at a concentration of 2×10^6 cells /ml, and 10^{-8} M bodipy-ouabain was added. Cells were then incubated for 20 min at 37°C and fluorescence was measured at 488 nm using a LSRII flow cytometer (BD Biosciences).

Sperm Motility Assays

To determine sperm motility under noncapacitating conditions, approximately 3×10^6 spermatozoa from wild-type and transgenic mice expressing rat α4 were resuspended in 300 μl of modified Tyrode medium, supplemented with 1.7 mM $CaCl_2$, which is required for sperm motility. To assess the hyperactive pattern of motility, typical of sperm capacitation, cells were incubated in modified Tyrode medium with the addition of 1.7 mM $CaCl_2$, 25 mM sodium bicarbonate, and 0.5% bovine serum albumin [25]. Cells were incubated at 37°C for different times and labeled with 2 μl of a 75 μM stock of the fluorescent nucleic acid stain SITO 21, which helps in tracking cell movement. After 2 min incubation with the dye, 7-μl aliquots from each sample were taken and placed into a 20-μm-depth glass cell chamber (Leja Products B.V.). Chambers were viewed on an Olympus BX51 microscope through a 20× phase objective and maintained at 37°C on a heated platform. Viewing areas on each chamber were captured using a CCD camera. Samples were analyzed by computer-assisted sperm analysis (CASA), using the Minitube SpermVision Digital Semen Evaluation system (version 3.5; Penetrating Innovations). Different sperm motility parameters were analyzed, including total motility; progressive motility; curvilinear, average path, and straight-line velocities; and amplitude of lateral head displacement. The analytical setup parameters used considered a cell identification or cell size area between 5 and 900 μm^2, a cutoff velocity corresponding to a minimum average orientation change of the head of 6°, and a progressive motility threshold corresponding to a straight-line distance of more than 5 μm. Linearity was calculated from the ratio between straight-line velocity and curvilinear velocity during the measurement period. Amplitude of lateral head displacement was obtained as the maximum distance of the sperm head from the average trajectory of the sperm during the analysis period. Hyperactive motility was defined as the sperm displaying a curvilinear velocity of more than 78 μm/sec, a linearity of less than 65%, and an amplitude of lateral head displacement of more than 6.5 μm. An average of 80 cells/field was

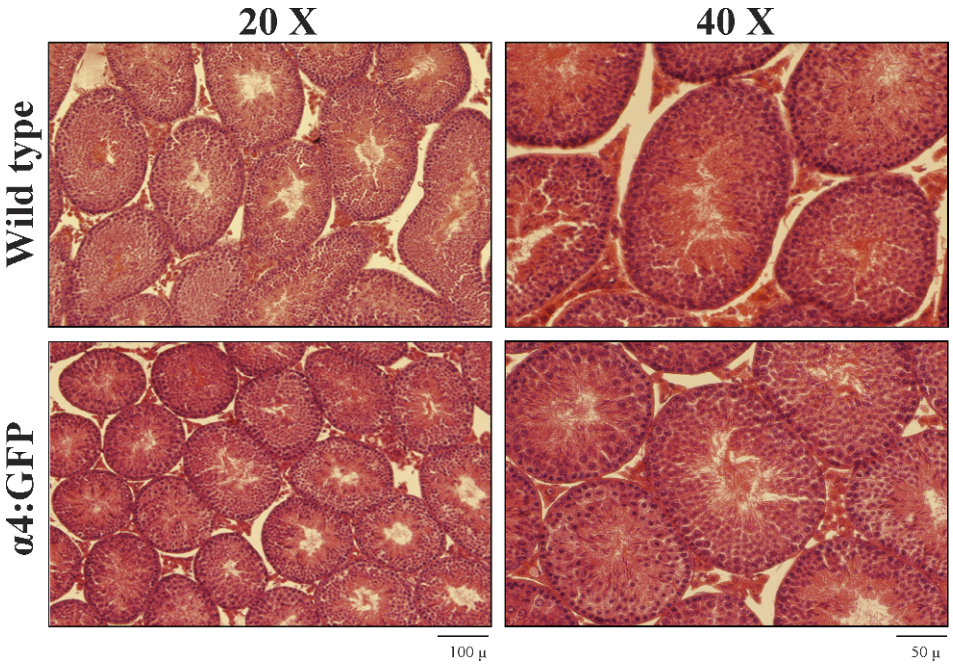

FIG. 1. Testis sections from transgenic mice expressing the rat Na,K-ATPase α4 isoform have similar morphology to testis from wild-type mice. Testis tissue was dissected, fixed, embedded in paraffin, and sectioned in 10-μm-thick sections. After staining with hematoxylin-eosin, samples were subjected to microscopy, using 20× and 40× objectives. Bars = 100 μm and 50 μm for the ×20 and ×40 magnifications, respectively.

captured, at a rate of 30 frames per field, and a total of 10 fields in each sample were analyzed. Each field was taken randomly, scanning the slide following a preestablished path to ensure consistency in the method.

Sperm Membrane Potential Assays

Membrane potential was determined using the fluorescent indicator $DiSC_3(5)$ (3,3′-dipropylthiodicarbocyanine) as previously described [28–30]. Sperm samples containing 10×10^6 cells/ml were resuspended in modified Tyrode medium supplemented with 1.7 mM $CaCl_2$ and were treated with $DiSC_3(5)$, at a final concentration of 1 μM for 3 min at 37°C. Sperm was further incubated for another 2 min with m-chlorophenyldrazone at a final concentration of 1 μM to block mitochondrial membrane potential [23, 31]. After this time period, 2.5 ml of the suspension was transferred into a cuvette arranged with gentle stirring and maintained at 37°C. Fluorescence of the samples was recorded at an excitation/emission wavelength of 620/670 nm. After determining fluorescence of the different experimental conditions, calibration of the fluorescence changes into mV was performed in the same sample, by adjusting the membrane potential of the cells as previously described [28]. Briefly, the K^+ ionophore valinomycin was added (1 μM final concentration) to allow K^+ to equilibrate across the plasma membrane. Then, the K^+ concentration was increased stepwise, to a final concentration in the media of 9.9, 13.9, 21.9, and 37.9 mM KCl. Membrane potential can be calculated from the distribution of K^+ in the cells, which follows the Nernst equilibrium. The membrane potential for the different KCl amounts added corresponded to −80, −67, −58, −45, and −30 mV, respectively. The experimental sperm membrane potential in each case was linearly interpolated using these data as plasma membrane potential versus arbitrary units of fluorescence as described [23, 28–30].

Acrosome Reaction Assays

Approximately 2×10^6 spermatozoa from wild-type and transgenic mice were resuspended in modified Tyrode medium with 1.7 mM $CaCl_2$, 25 mM sodium bicarbonate, and 0.5% bovine serum albumin. Samples were treated with progesterone at a final concentration of 10 μM to induce sperm acrosome reaction. After incubation for different times at 37°C, cells were centrifuged and washed once with PBS. Then, sperm was fixed with 100 μl of absolute methanol for 2 min at −20°C, were smeared on glass slides, and were let air dry. Sperm was stained using a 1:1500 dilution of fluorescein-labeled peanut agglutinin, FITC-PNA (Vector Laboratories), in PBS for 10 min at 37°C. Slides were washed three times for 5 min each with PBS, were layered with Slowfade mounting solution with DAPI stain (Invitrogen), and were covered with coverslips. Samples were examined under a fluorescent microscope for the presence of FITC-PNA label. A minimum of 350 cells were scored for each time point per experiment. Acrosome-reacted spermatozoa without FITC-PNA label were expressed as percentage of the total amount of cells labeled with DAPI.

Statistical Analysis

Experiments were repeated at least three times using a minimum of triplicate determinations. Statistical significance of differences between wild-type controls and α4 expressing transgenic samples was determined by the Student *t*-test, using Sigma Plot software (Jandel Scientific). Statistical significance was defined as $P < 0.05$.

RESULTS

Na,K-ATPase α4 Isoform Expression in Transgenic Mice

To study the biological effects of the Na,K-ATPase α4 isoform in sperm movement, we engineered transgenic mice expressing the Na,K-ATPase α4 isoform fused to GFP. To obtain specific expression of this transgene in male germ cells, we placed the *Atp1a4*-GFP construct downstream of the protamine 1 promoter. This promoter directs the expression of various transgenes in male germ cells at late stages of spermatogenesis and after male germ cell meiosis [32–34]. Our previous work on the ontogeny of the α4 isoform during spermatogenesis has determined that expression of α4 is up-regulated postmeiotically, increasing in spermatids and reaching the highest levels in spermatozoa [17]. Thus, the protamine 1 promoter we used for expressing *Atp1a4*-GFP had the potential to direct the synthesis of the transgene in male germ cells in a fashion that is temporally similar to that of the native male germ cells. This allows for the study of the α4 isoform in the already differentiated male gamete.

After confirming positive incorporation of *Atp1a4*-GFP in mice, two lines were established that were phenotypically identical. These transgenic mice appeared overall normal and they did not show differences in testis size or general morphology compared to wild-type mice (data not shown). Also, we did not observe histological differences between transgenic and wild-type mice (Fig. 1). In addition, these animals presented normal fertility. The number of mice/litter was 7.9 ± 0.4 for transgenic mice compared to 7.0 ± 1.5 for wild-type mice, and the intervals between litters was 22.3 ± 0.5 and 30.0 ± 7.0 days for transgenic and wild-type mice, respectively.

To determine whether the rat *Atp1a4* transgene was appropriately expressed at the protein level, we used

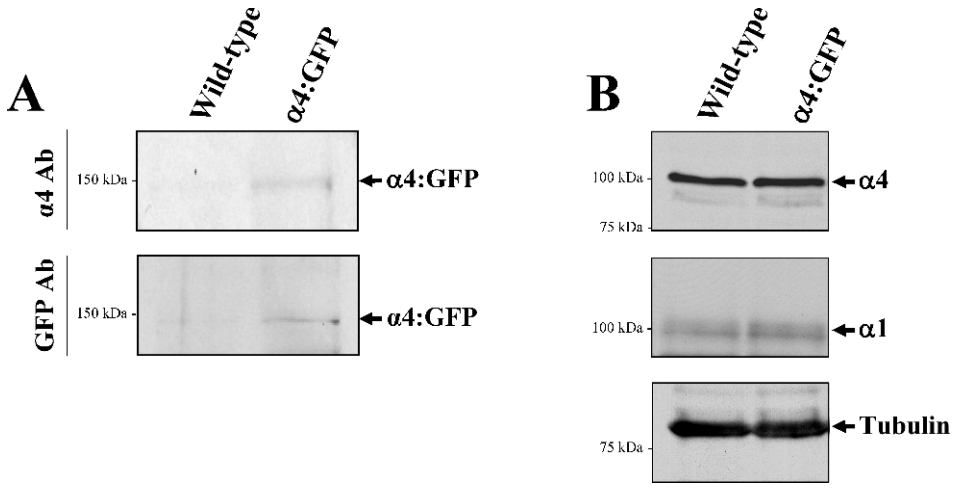

FIG. 2. The rat Na,K-ATPase α4 isoform fused to GFP is expressed in transgenic mice. **A**) Expression of α4:GFP. The α4:GFP transgene expression was determined in testis from wild-type and transgenic mice membrane fractions. Immunoblot analysis was performed using a chicken-generated anti-α4 antiserum or anti-GFP polyclonal antibody. **B**) Expression of the endogenous mouse Na,K-ATPase α4 and α1 isoforms. The α4 polypeptide was detected using the anti-α4 antiserum mentioned above and α1 was identified with C464-CB antibody. Tubulin is shown as a loading control. In both **A** and **B**, secondary antibodies conjugated to horseradish peroxidase and chemiluminescence were used for detection.

immunoblot analysis of testis proteins using antibodies against the α4 and GFP polypeptides. As shown in Figure 2A (top panel), an antibody against α4, which we had previously generated in chicken [18], showed expression of a protein with a molecular weight corresponding to the fused α4:GFP polypeptide in samples from the transgenic animals, but not in those from wild-type mice. The presence of α4:GFP was also identified with an anti-GFP antibody only in sperm from the rat *Atp1a4* transgenic mice (Fig. 2A, bottom panel). In addition, we studied expression of the endogenous mouse α4 and α1 polypeptides, the only two Na,K-ATPase α isoforms expressed in testis [10]. As shown in Figure 2B, our anti-α4 antiserum produced in chicken and an anti-α1 antiserum detected similar levels of the mouse α4 and α1 isoforms in wild-type and transgenic mice samples. As a loading control, the housekeeping protein tubulin was used (Fig. 2B). These results show that the rat α4 polypeptide is expressed in transgenic mice and that this does not interfere with the expression levels of the endogenous α4 and α1 isoforms.

To determine specific expression of α4:GFP in spermatozoa, we performed immunocytochemistry of sperm taken from the cauda of the epididymis. For this, we used a polyclonal antiserum against α4 that we have prepared, which recognizes the Na,K-ATPase α4 polypeptide from rat, but shows little cross-reactivity with the endogenous mouse α4 isoform in immunocytochemistry. As shown in Figure 3A, this anti-α4 antibody labeled spermatozoa only in transgenic mice expressing the rat α4; it did not produce significant signal in sperm from wild-type mice containing the native mouse isoform. The transgene-expressed α4 polypeptide was localized to the sperm flagellum, extending throughout the length of the sperm tail. A similar expression pattern for the α4:GFP polypeptide was observed when the anti-GFP antibody was used (Fig. 3B). Altogether, these results indicate that the rat α4:GFP protein is expressed in transgenic mice sperm, where it is present along the sperm flagellum.

Having established the expression of the transgene, we next determined whether the rat α4 isoform was functional in transgenic mice. Thus, we measured Na,K-ATPase activity in spermatozoa from wild-type and transgenic mice. To distinguish the activity of the α4 isoform from that of the ubiquitous α1 polypeptide, we took advantage of the vast difference in ouabain sensitivity that characterizes these isoforms. As reported in previous studies on the kinetic properties of the Na,K-ATPase isoform from rodents [10], the inhibition constant for ouabain is much higher for the α4 than for the α1 isoform, with values in the nanomolar and micromolar range respectively [17]. Based on this property, we measured Na,K-ATPase activity in sperm samples from wild-type and transgenic mice at ouabain concentrations that we had previously determined to be adequate to differentiate the activity of α4 from α1. We used 10^{-6} M ouabain, which completely inhibits activity of α4 and has no significant effect on α1, and 10^{-3} M ouabain, which inactivates both the α1 and α4 isoforms [17]. In this manner, by subtracting the Na,K-ATPase activity measured at 10^{-6} M from that in the absence of ouabain, the hydrolysis of ATP corresponding to the α4 isoform can be calculated. On the other hand, the difference in activity between samples containing 10^{-6} M and 10^{-3} M ouabain reflects the hydrolysis of ATP dependent on α1. The

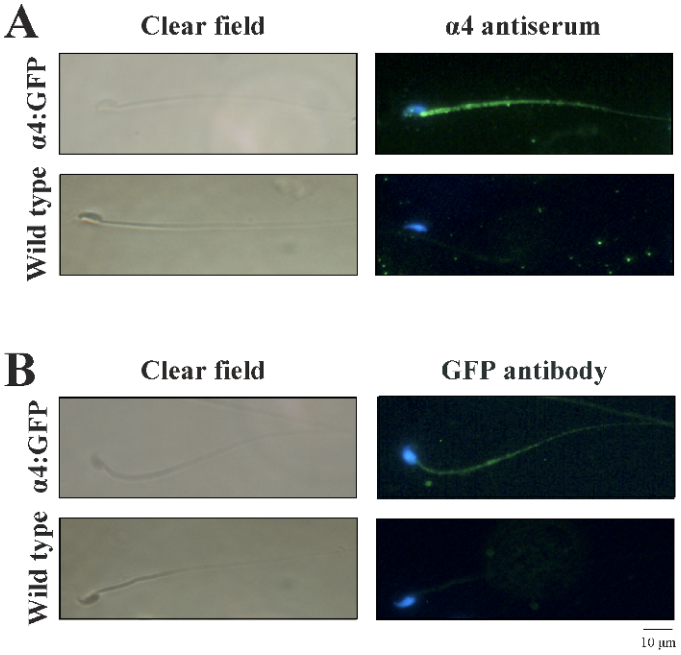

FIG. 3. The rat Na,K-ATPase α4 localizes to the sperm flagellum of transgenic mice sperm. Spermatozoa were isolated from the cauda of the epididymis and were plated and attached onto glass coverslips. Then, cells were fixed, permeabilized, and subjected to immunocytochemistry. Samples were incubated with a rat-specific anti-α4 antiserum generated in rabbits (**A**) or with an anti-GFP polyclonal antibody (**B**). After washing, spermatozoa were treated with Alexa Fluor 488-conjugated secondary antibodies. Samples from wild-type mice were used as a control. DAPI was included to stain the cell nuclei. Bar in the bottom right = 10 μm.

total Na,K-ATPase activity in spermatozoa from mice expressing rat α4 was significantly higher than in wild-type sperm (Fig. 4A, bars labeled Total). In addition, separate analysis of the α4 and α1 isoforms indicated that the augment in Na,K-ATPase was due to the selective increase in ATP hydrolysis dependent on α4 (Fig. 4A, bars labeled α4). In contrast, activity of the α1 isoform remained unchanged in sperm from wild-type and transgenic mice (Fig. 4A, bars labeled α1). These results suggest that the expressed α4 transgene is catalytically competent in the host environment of mouse sperm.

Further indication of the functional capability of the expressed rat α4 isoform was obtained through ouabain-binding studies. For this, we measured binding of the ouabain fluorescent derivative bodipy-ouabain in spermatozoa from wild-type and α4 transgenic mice. To selectively assess ouabain binding by the α4 isoform, we used particularly low concentrations of bodipy-ouabain (10^{-8} M), which assures binding to only the high-affinity α4 isoform, and not to α1 [19]. As shown in Figure 4B, sperm from transgenic mice showed an increase of approximately 40% in bodipy-ouabain binding compared to sperm from wild-type animals. Altogether, the results presented in Figure 4, A and B, show that the rat α4 polypeptide produced in transgenic mice is functional and that it displays the typical properties of the α4 isoform, including its ability to hydrolyze ATP in a highly ouabain-sensitive manner and its capacity to bind ouabain with high affinity.

In a previous report, we demonstrated that inhibition of α4 activity with ouabain caused depolarization of the male gamete, and this was interpreted as the ability of the α4 isoform in maintaining sperm membrane potential [20]. Here, we investigated the effect of increased expression of α4 in the membrane potential of spermatozoa. For this, we determined membrane potential in sperm from wild-type and transgenic mice, using the fluorescent indicator DiSC3(5) as described [23]. Figure 5A shows representative traces for the fluorescence measured in the wild-type and transgenic samples. The initial segments in the recordings represent the fluorescent emission that corresponds to the membrane potential of the cells, followed by a calibration curve, obtained after addition of the ionophore valinomycin and increasing amounts of KCl. Figure 5B presents the calculated values of membrane potential

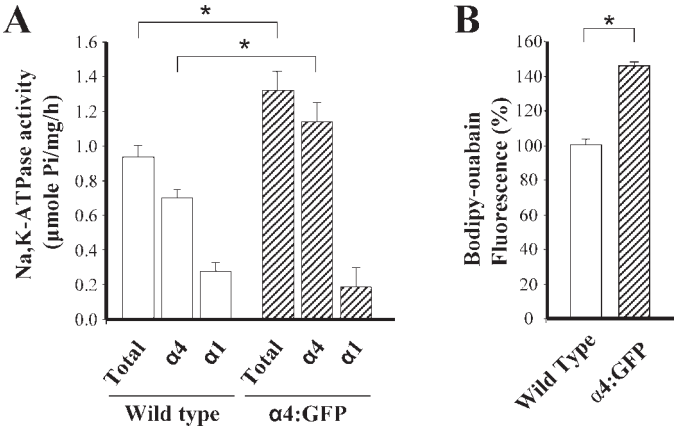

FIG. 4. The Na,K-ATPase α4 isoform expressed in sperm from transgenic mice is functional. Caudal epididymal sperm was collected, homogenized, and used to measure Na,K-ATPase activity, or the whole cells were used to determine bodipy-ouabain binding. (**A**) Na,K-ATPase assays. Activity of the total Na,K-ATPase in the cells was determined as the Na^+- and K^+-dependent hydrolysis of ATP sensitive to 10^{-3} M ouabain. Activity of the α4 isoform was identified as that sensitive to 10^{-6} M ouabain, whereas activity of α1 was calculated as the difference in specific ATP hydrolysis between 10^{-6} M and 10^{-3} M ouabain. Bars represent the mean ± SEM obtained from 4 (wild-type) and 5 (transgenic) experiments performed in quadruplicate. Asterisks indicate significantly different values, $*P < 0.05$. (**B**) Ouabain-binding assays. Sperm from wild-type and transgenic mice were labeled with the fluorescent ouabain derivative bodipy-ouabain. After 20 min incubation, samples were subjected to flow cytometry and levels of fluorescence were determined at an excitation of 488 nm. Bars represent the mean ± SEM of three experiments. Values significantly different from the wild type are indicated, $*P < 0.001$.

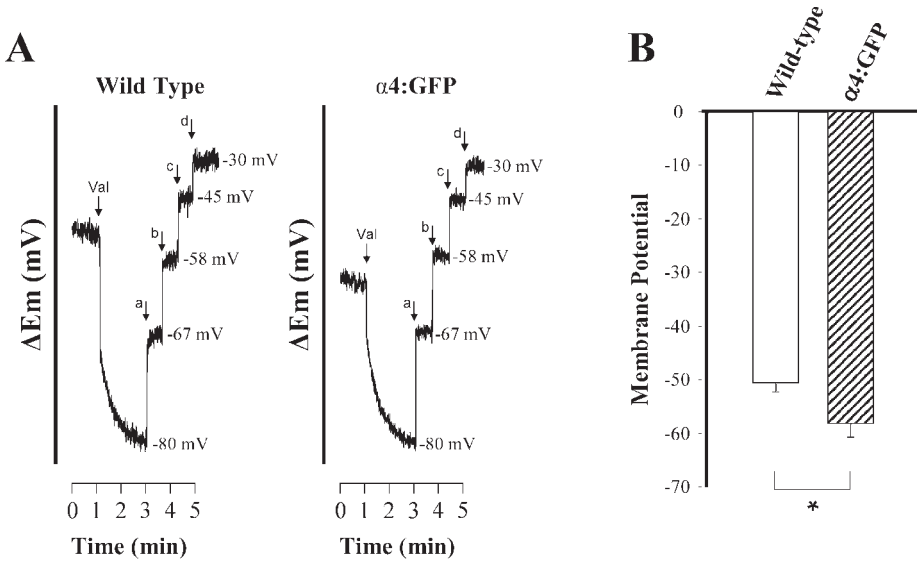

FIG. 5. The plasma membrane of sperm from transgenic mice expressing the Na,K-ATPase α4 isoform is hyperpolarized. Epididymal sperm from wild-type and transgenic mice was isolated in modified Tyrode medium and membrane potential of the cells was determined using the fluorescent indicator DiSC3(5). (**A**) Representative traces are shown, obtained at an excitation/emission wavelength of 620/670. Calibration was performed with the addition of valinomycin (indicated in the graph with an arrow [Val]), followed by the sequential addition of increasing amounts of KCl. The initial medium contained 5.9 mM K^+ and the subsequent KCL additions (indicated with the letters a to d), resulted in the following final KCL concentrations in mM: (a) 9.9, (b) 13.9, (c) 21.9, and (d) 37.9, which corresponded to the indicated membrane potentials (ΔEm) in mV respectively. (**B**) Compiled data from three different experiments each performed in triplicate. Bars represent the mean ± SEM. Statistical significance is indicated with an asterisk, $*P = 0.03$.

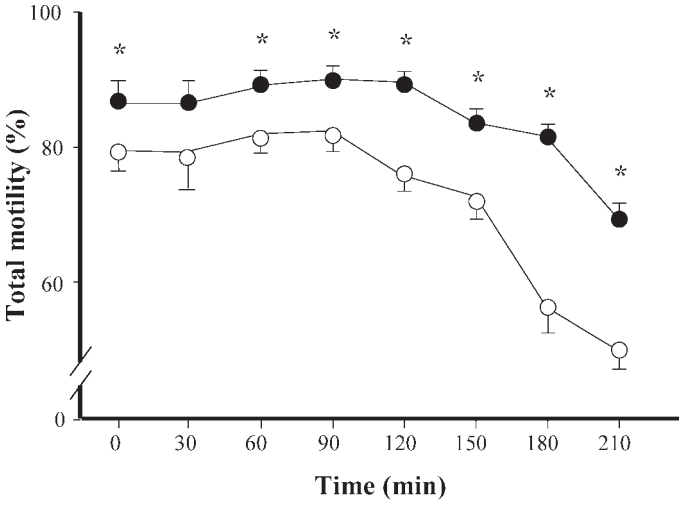

FIG. 6. Expression of rat α4 enhances sperm motility in transgenic mice. Sperm from wild-type and transgenic mice was collected in modified Tyrode medium with the addition of calcium and incubated at 37°C for the indicated times. Motility was determined using CASA. Symbols represent wild-type (white circles) or transgenic mouse sperm (black circles). Each value is the mean and bars show the standard errors of the mean of three experiments. Values significantly different from the wild type are indicated, $*P < 0.001$.

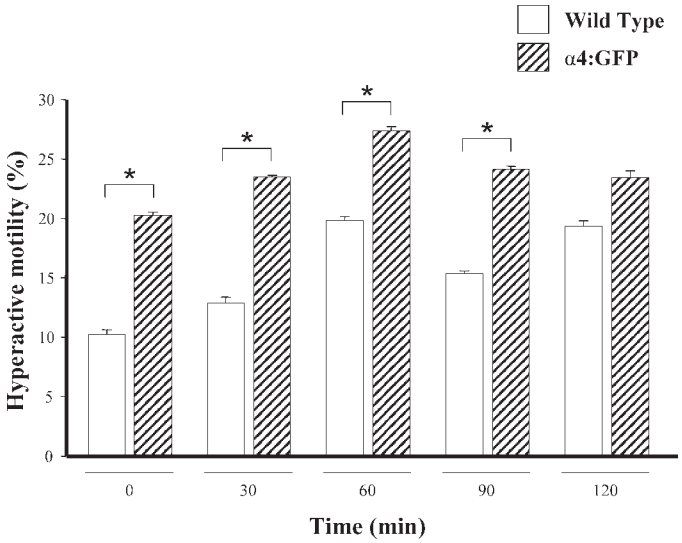

FIG. 8. Expression of rat α4 enhances sperm hyperactive motility in transgenic mice. Sperm from wild-type and transgenic mice was incubated in modified Tyrode medium supplemented with calcium, bicarbonate, and bovine serum albumin to support capacitation, and samples were incubated at 37°C for the indicated times. Hyperactive motility was determined using CASA. Bars represent wild-type (open bars) or transgenic mouse sperm (striped bars). Each value is the mean and bars represent the SEM of six experiments. Values significantly different from wild type are indicated, $*P < 0.001$.

for each sample, averaged from different experiments. As shown, compared to wild-type samples, increased expression of α4 resulted in hyperpolarization of the sperm plasma membrane. This suggests that the increase in activity of the rat α4 isoform is physiologically relevant, and that the uneven Na^+ and K^+ transport catalyzed by α4 creates the ion gradients that are necessary in determining sperm membrane potential.

Pharmacological inhibition of Na,K-ATPase α4 isoform activity has suggested a role for α4 in sperm motility [16, 18, 20]. To determine whether the expressed rat α4 isoform has any consequence in sperm movement, we determined sperm motility in wild-type and transgenic mice during a 3.5-h period using CASA. As shown in Figure 6, total motility of spermatozoa from wild-type mice exhibited maximal values for the first 90 min, and then cell movement slowly decreased with time, as expected after incubation of sperm for relatively long periods of time (open circles in Fig. 6). A similar time course for motility was found in sperm from transgenic mice. However, different from wild-type sperm, spermatozoa expressing the rat α4 exhibited significantly higher motility at most time points tested (closed circles in Fig. 6). These experiments confirmed the role of α4 in sperm motility and showed that increased expression of α4 enhances total sperm movement, providing the cells with the capacity to move for longer periods of time.

To further study the role of α4 in sperm motility, other parameters of sperm movement were also analyzed by CASA. As shown in Figure 7, sperm from transgenic mice exhibited an increase in a series of parameters of sperm motility, including progressive motility; straight-line, curvilinear, and average path

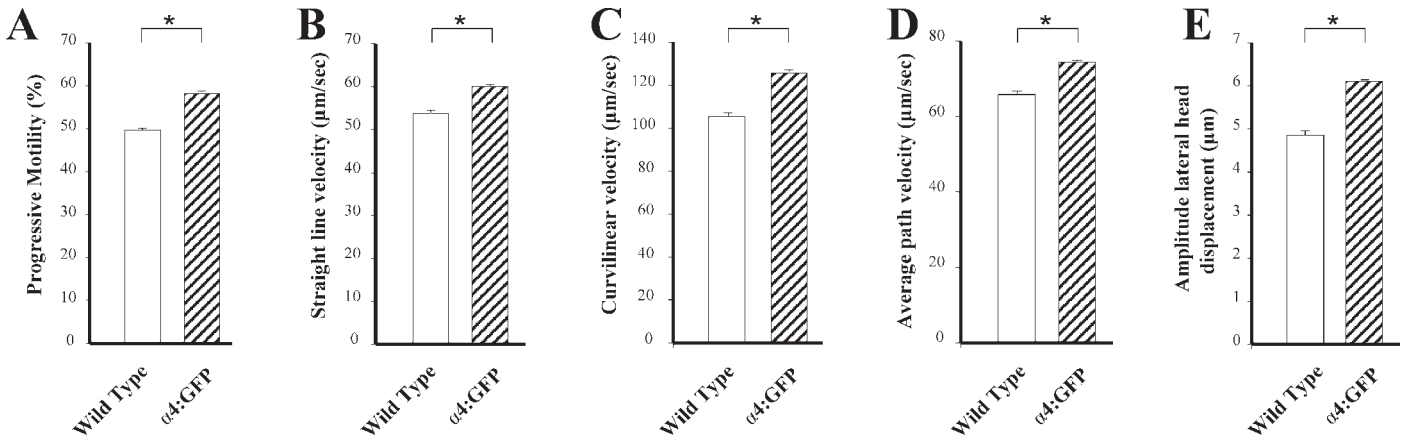

FIG. 7. The rat Na,K-ATPase α4 isoform increases several parameters of motility in sperm from transgenic mice. Spermatozoa from the cauda of epididymides were isolated in modified Tyrode medium. Sperm motility was determined using CASA and different parameters of sperm movement were analyzed after 1 h incubation using the SpermVision system. (**A**) Progressive motility, (**B**) straight-line velocity, (**C**) curvilinear velocity, (**D**) average path velocity, and (**E**) amplitude of lateral head displacement. Bars represent the mean ± SEM of three experiments. Bars represent wild-type (open bars) or transgenic mouse sperm (striped bars). Values significantly different from the control are indicated with an asterisk, with *P* values ranging between <0.05 and <0.001.

velocities, and amplitude of lateral head displacement (Fig. 7, A–E). Altogether, these results suggest that activity of the Na,K-ATPase α4 isoform is important in supporting sperm flagellar movement, and that up-regulation of its expression enhances many aspects of sperm movement.

The experiments shown above have been performed under conditions that do not support sperm capacitation. Before spermatozoa can fertilize the egg, they must undergo capacitation, a process that is characterized by a series of changes in the cells, including specific changes in sperm motility, known as the hyperactivated pattern of motility [35]. To determine whether expression of the α4 isoform plays a role in the capacitation dependent hyperactive motility, we incubated wild-type and transgenic sperm for different times in modified Tyrode medium supporting capacitation and measured hypermotility using CASA. As shown in Figure 8, sperm from wild-type mice showed little hyperactive motility before capacitation, and this increased after incubation under capacitating conditions in a time-dependent manner as expected [35]. Similar results were found for sperm from the transgenic mice; however, in these cells, hypermotility was significantly higher than that of the wild-type mice at all time points during the first 90 min of incubation. Although still higher for sperm from transgenic mice, hypermotility values were not significantly different from those of wild-type sperm at 120 min. Interestingly, hyperactive motility in the transgenic sperm approximately doubled that of the wild-type cells before capacitation (time 0). Altogether, these data suggest that activity of the Na,K-ATPase α4 isoform not only is important in supporting sperm flagellar movement under noncapacitated conditions, but also contributes to the hyperactive motility of sperm that is characteristic of sperm capacitation. In addition, increased expression of α4 appears to endow sperm with the ability to prematurely initiate its hyperactive pattern of motility.

To examine whether increased expression of the α4 isoform is able to regulate other aspects of sperm capacitation besides hyperactivation, we determined the development of the acrosome reaction in the cells, a parameter that has been commonly used as an overall indicator of sperm capacitation [36]. For this, we incubated spermatozoa from wild-type and transgenic mice with progesterone in media supporting capacitation to induce the acrosomal reaction and measured the acrosomal status of the cells. As shown in Figure 9, acrosomal exocytosis increased in both wild-type and transgenic sperm in a time-dependent manner; however, no significant differences were observed between the wild-type and transgenic samples at the times studied. These results suggest that the increase in α4 expression is not involved in the events that lead to the release of the acrosome content from the cells.

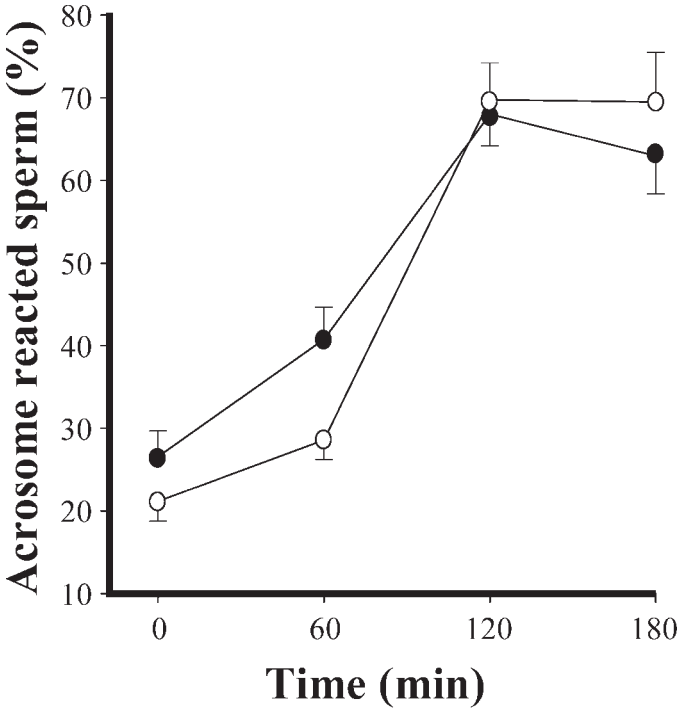

FIG. 9. Na,K-ATPase rat α4 expression does not alter progesterone-induced acrosome reaction in transgenic mice. Sperm from wild-type and transgenic mice was incubated in modified Tyrode medium supplemented with calcium, bicarbonate, and bovine serum albumin in the presence of progesterone to induce acrosome reaction. After incubation at 37°C for the indicated times, the acrosome status of the cells was measured with FITC-PNA. Symbols represent wild-type (white circles) or transgenic mouse sperm (black circles). Each value is the mean and bars show the SEM.

DISCUSSION

In this work, we have determined the biological role of the Na,K-ATPase α4 isoform by using transgenic mice that express the rat homolog of this isoform in spermatozoa. This approach provided a new alternative to study the biological relevance of α4 that is distinct from our previous in vitro studies, in which we exploited the unique high affinity of α4 for ouabain to investigate the function of this isoform after pharmacological inhibition of its activity [16, 17]. Testis and spermatozoa from the transgenic mice we generated showed normal morphology and successfully expressed the rat Na,K-ATPase α4 isoform. As shown from our immunoblot analysis, the expression levels of the rat α4 appear to be relatively modest and the amount of the rat α4 transgene is lower than that of the endogenously expressed mouse α4 isoform. This suggests that the protamine 1 promoter is not particularly strong in driving the expression of the Na,K-ATPase α4 isoform. Alternatively, the relatively low expression levels of rat α4 may depend on the mechanisms that control the synthesis and/or degradation of α4, which limit the total amounts of this isoform expressed in male germ cells. In any case, the levels of rat α4 expressed in the transgenic mice are sufficient to induce phenotypic changes in the host spermatozoa.

Our immunocytochemical analysis confirmed expression of the rat α4 isoform and showed that this polypeptide is distributed to the flagellum of transgenic spermatozoa. This localization concurs with previous observations showing that the α4 isoform is mainly present in the sperm tail [15, 17, 18]. However, compared to previous reports, which indicate that α4 is primarily confined to the midregion of the wild-type sperm flagellum [16–18], expression of the rat α4 transgenic protein is less limited to the middle piece and extends throughout the sperm tail of transgenic mice. This broader distribution may reflect the inability of the mechanisms involved in protein targeting in transgenic mice male germ cells to accommodate the excess α4 polypeptide produced in these cells.

Concurrent with expression of the α4 isoform, we found an increase in total Na,K-ATPase activity in spermatozoa from the transgenic mice. Moreover, as shown by selective inhibition of ATP hydrolysis with low concentrations of ouabain, we determined that the Na,K-ATPase activity that augmented in sperm from the transgenic mice exhibited the high ouabain sensitivity profile characteristic of the α4 isoform [11]. In

addition, we found that sperm from the transgenic mice displayed high specific binding of bodipy-ouabain, further indicating that the augmented Na,K-ATPase in transgenic mouse sperm corresponded to that of the expressed *Atp1a4* transgene. These results confirm previously published data showing that the Na,K-ATPase α4 isoforms from rat and mouse are similar, with respect to both their structure [37] and their functional properties [12, 15, 19]. Also, our activity assays show that the incorporation of GFP at the 3′ end of *Atp1a4* did not alter the activity and functional properties of this Na,K-ATPase isoform, suggesting that the phenotype we found in the sperm from the transgenic animals depends on the function of the expressed α4 polypeptide. Because bodipy-ouabain binding assays were performed in whole cells, and ouabain can only bind to extracellular domains in Na,K-ATPase [1], the higher bodipy-ouabain binding we found in transgenic sperm indicates that the rat α4 polypeptide is present at the plasma membrane of the cells. This shows that the rat α4 isoform is targeted to the surface of the mouse host cells, where its activity is relevant to the physiology of sperm. Altogether, these results indicate that the rat α4 produced in the transgenic mice spermatozoa is catalytically competent and functional.

In contrast, sperm expressing the *Atp1a4* transgene exhibited no change in the expression and activity of the Na,K-ATPase α1 isoform. This constancy in α1, without a compensatory down-regulation due to the overexpression of α4, suggests that functional regulation of each of the Na,K-ATPase α polypeptides is controlled independently. In addition, this suggests that activity of α1 and α4 are not interchangeable and that these isoforms perform specific functions. In this respect, we have previously shown that the enzymatic and transport properties and the localization and function of α1 and α4 are distinct [10, 17, 19]. Thus, specific inhibition of the α4 isoform, but not the α1 isoform, with ouabain affects several cell parameters that are important for sperm motility [20]. It is possible, then, that although the widely expressed α1 isoform may be regulating the basal Na^+ and K^+ homeostasis, the α4 polypeptide works to further maintain the ion gradients necessary for sperm motility. The in vivo results from our present work support the notion that the α1 and α4 isoforms are playing different roles in the male gamete, and the latter is important for sperm motility.

The Na^+ and K^+ transport catalyzed by the Na,K-ATPase maintains an uneven distribution of electrical charges across the plasma membrane, and this contributes to maintaining cell membrane potential [3]. Our results indicate that increased expression of α4 in transgenic sperm causes a shift in membrane potential, with hyperpolarization of the cell plasma membrane. This agrees with results from our previous report showing that inhibition of α4 with ouabain affects sperm membrane potential, depolarizing the cells [20]. The correspondence between activity levels of the α4 isoform and changes in sperm membrane potential from either overexpression of α4 (the present study) or inhibition of α4 with ouabain [20] suggests the importance of the α4 isoform in being the main Na,K-ATPase isoform involved in maintaining the membrane excitability of spermatozoa. The dependence of sperm membrane potential on the α4 isoform and not the α1 isoform is further supported by our Na,K-ATPase assays, which show changes in activity of only the α4 and not the α1 polypeptide in the transgenic mice. In addition, as previously shown, α1 has less influence in the transmembrane electrical charges of spermatozoa than α4 [20]. In this manner our results indicate that α4 is the Na,K-ATPase isoform that plays the major role in sustaining the asymmetrical distribution of ions across the plasma membrane, which is essential in maintaining sperm membrane potential.

Proper membrane excitability is essential to sperm motility, and this could be one of the mechanisms by which α4 sustains membrane sperm motility [20]. Specific inhibition of α4 with ouabain has been shown to impair total sperm motility and a variety of parameters of sperm motility [16, 18, 20]. Our current data provides direct evidence that expression and activity of α4 in transgenic mice is associated not only with increases in total sperm motility, but also progressive motility; straight-line, curvilinear, and average path velocities; and lateral head displacement. Interestingly, increased expression of α4 also enhanced sperm hypermotility after incubation of the cells under capacitating conditions for various times. This indicates that activity of the α4 isoform is important for the hyperactive pattern of sperm motility that is characteristic of sperm capacitation and essential for sperm fertility [35]. Moreover, sperm from transgenic mice showed higher hyperactive motility even before incubation in capacitating medium. This, along with the fact that differences in sperm hypermotility diminished between wild-type and transgenic samples at 120 min incubation in capacitating medium, suggests that increased expression of the α4 isoform is able to induce premature sperm hyperactivation. Altogether, the influence of α4 activity in multiple parameters of sperm movement, under both noncapacitating and capacitating conditions, highlights the relevance of α4 in sperm fertility. In this regard, transgenic mice expressing rat α4 exhibit a normal breeding phenotype. This is based on our in vivo mating experiments, which cannot distinguish increases in sperm fertility because the offspring produced is limited by the female factor. Further experiments will clarify whether sperm expressing increased levels of α4 have increased fertilizing capability. In any case, our findings expand our previous data regarding the importance that activity of α4 has in various motion components, which are all required for normal sperm motility.

Our results also show no significant differences in the acrosomal reaction induced by progesterone in sperm from wild-type and transgenic mice. Thus, whereas α4 is important for the hyperactive pattern of motility accompanying sperm capacitation, and may force sperm into a partially capacitated state, it is not involved in the development of the sperm acrosomal reaction. Therefore, α4 may be a downstream effector, which is required for some (i.e., hyperactive motility) but not all events that constitute the complex process of sperm capacitation.

In conclusion, our work provides new evidence for the role of the Na,K-ATPase α4 isoform and shows that manipulation of the expression of the Na,K-ATPase α4 isoform is an important factor in the control of sperm motility. Additional studies are in progress to further understand how α4 isoform expression and activity can be modulated to control fertility of the male gamete.

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