Sperm motility is dependent on a unique isoform of the Na,K-ATPase

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Running Title: A biological role for the Na,K-ATPase α4 isoform

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A biological role for the Na,K-ATPase α4 isoform

The Na,K-ATPase, a member of the P-type ATPases, is comprised of two subunits, α and β, and is responsible for translocating Na\(^+\) out of the cell and K\(^+\) into the cell using the energy of hydrolysis of one molecule of ATP. The electrochemical gradient it generates is necessary for many cellular functions, including establishment of the plasma membrane potential and transport of sugars and ions in and out of the cell. Families of isoforms for both the α and β subunits have been identified and specific functional roles for individual isoforms are just beginning to emerge. The α4 isoform is the most recently identified Na,K-ATPase α isoform and its expression has only been found in testis. Here we show that expression of the α4 isoform in testis is localized to spermatozoa, and that inhibition of this isoform alone eliminates sperm motility. These data describe for the first time a biological function for the α4 isoform of the Na,K-ATPase, revealing a critical role for this isoform in sperm motility.

The Na,K-ATPase is a heteromeric, integral membrane protein that is responsible for the electrogenic translocation of three sodium ions out of the cell and two potassium ions into the cell using the energy of hydrolysis of one molecule of ATP (1-3). This enzymatic activity results in the production of an electrochemical gradient that is required for many cellular processes, including establishment of the resting membrane potential, regulation of the osmotic balance, and generation of the Na\(^+\) gradient necessary for the transport of many ions and other substrates across the plasma membrane (1-3). Structurally, the Na,K-ATPase consists of two subunits, the α subunit having a molecular weight of 112 kDa and the glycosylated β subunit having a protein molecular weight of 35 kDa (1-3). The α subunit is the catalytic subunit of the enzyme, containing the cation binding sites, the cardiac glycoside binding site, and the ATP binding site.
(2), while the β subunit is necessary for maturation of the enzyme, localization to the plasma membrane (4-7), and stabilization of the K⁺-occluded intermediate form of the protein (8-9). An additional protein, γ, has been recently described to be associated with the Na,K-ATPase in some tissues and appears to modulate the enzyme’s affinity for cations (10-12).

Isoforms for the α and β subunits have been identified, all exhibiting unique tissue and developmental expression patterns (3,13-16), and specific functional roles for each are now beginning to be defined (17). Studies focusing on the biochemical properties of the Na,K-ATPase carrying different α isoforms have revealed modest differences in enzymatic activity (18), however it is uncertain whether these differences have physiological significance. Therefore it is important to consider other properties of these isoforms in order to understand the reason for the existence of multiple Na,K-ATPase α isoforms. Recently, this laboratory has reported for the first time a unique functional role for the α2 isoform in Ca²⁺ handling in cardiac myocytes (17), highlighting the importance for examination of the biological function(s) performed by other tissue-specific α isoforms.

The tissue expression pattern of the Na,K-ATPase α4 isoform is one of the most restricted, having only been identified in testis of mouse, rat, and human, and at lower levels in mouse epididymis (13,17,19). Biochemical characteristics of the α4 isoform have been recently reported, revealing it to be a high affinity ouabain receptor that also has a high affinity for both Na⁺ and K⁺, and exhibits Na⁺ and K⁺ stimulated, ouabain inhibitable ATPase activity (20,21). In order to better understand the unique functional role of the α4 isoform, we report here an in-depth analysis of this Na,K-ATPase isoform in rat. Our experiments show that it is expressed specifically in the midpiece of the flagellum of mature sperm cells and that inhibition of the α4
isoform alone eliminates sperm motility, demonstrating for the first time a critical role for this isoform in normal sperm function.

EXPERIMENTAL PROCEDURES

RNA Isolation and Northern Blot Analysis - Total cellular RNA was isolated by the guanidine thiocyanate method (Tri-Reagent, Molecular Research Center, Inc., Cincinnati, OH). Total RNA samples (10 µg/sample) were denatured in 1 M glyoxal, 54% DMSO and 0.01 M sodium phosphate buffer (pH 6.8), separated using a 1% agarose gel in 0.01 M sodium phosphate buffer, and then transferred to Sure Blot® Nylon Membrane (Intergen Company, Purchase, NY). Northern blots were screened for expression of α isoforms of the Na,K-ATPase using isoform-specific probes (17). Quantitation of mRNA levels was determined by exposing [32P]-labeled blots to a phosphor screen. Phosphor screens were scanned using a Storm 840 scanner and analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Densitometry results were corrected to levels of GAPDH and are reported as mean volume integrated values ± the standard error (SE) between samples from three animals.

Microsome Preparation, SDS-PAGE and Western Blot Analysis - Microsomes, SDS-PAGE and western blots were performed as previously described (17,20) using the following dilutions of primary antibody: 0.5 µg/ml of αb4 (20); 1:1000 of α1 isoform-specific monoclonal α6F (University of Iowa Developmental Hybridoma Bank, Iowa City, IA); 1:500 of α2 isoform-specific monoclonal antibody McB2 (generous gift from K. Sweadner); and 1:1000 of α3 isoform-specific monoclonal antibody (Affinity Bioreagents, Inc., Golden, CO). Quantitation of protein expression levels was performed using scanned western blots and ImageQuant software.
A biological role for the Na,K-ATPase α4 isoform (Molecular Dynamics). Densitometry results are reported as mean volume integrated values ± the standard error (SE) between samples from three animals.

Immunohistochemical Analysis of Testis - Testes were harvested from Sprague-Dawley rats (Harlan, Indianapolis, Indiana) and immediately placed in HistoPrep™ Buffered 10% Formalin (Fisher Scientific, Pittsburgh, PA) overnight at 4°C. Tissues were washed three times in PBS, dehydrated in ethanol and stored in 80% ethanol until paraffin embedding. Tissue sections were cut at a thickness of 8 to 10 microns, fixed to glass slides (Fisher Premium Brand glass slides, Fisher) and stored at 4°C until further use.

Immunohistochemical staining of testis sections was performed as follows. Tissue sections were deparaffinized by washing in HemoD (Fisher) three times, rehydrated in a series of ethanol washes from 100-70% and then placed in PBS. Slides were then incubated in methanol containing 0.5% hydrogen peroxide for removal of endogenous peroxidase activity. Tissue sections were blocked for nonspecific binding by incubation in PBS, pH 7.4, containing 0.2% Triton X-100 and 2% normal goat serum for one hour, then placed in a solution containing αb4 (5.0 µg/ml) overnight at 4°C. In order to observe secondary antibody interactions with tissue sections, additional slides were incubated overnight in the absence of primary antibody. The next day, sections were washed (0.1 M PBS containing 0.2% Triton X-100) and then exposed to the biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) for one hour at room temperature. At the end of the hour, sections were washed before incubation with an avidin-biotin complex (Vector Labs) for 30 minutes at room temperature. Slides were washed one final time, rinsed briefly in 0.1 M acetate buffer (pH 6.0), incubated with diaminobenzidine for 4 minutes, rinsed briefly in tris buffer (pH 7.6), incubated in a tris-cobalt solution (pH 7.2) for 4 minutes and then placed in deionized water. Stained tissue sections were dehydrated in a
series of ethanol washes from 70-100% and coverslips were mounted using Permoun (Fisher) diluted 1:1 with xylene.

*Immunocytochemical Analysis of Sperm* - The whole epididymis was removed from adult (8-12 weeks of age) Sprague-Dawley rats (Harlan) and placed in PBS. Epididymis tubules were carefully minced and sperm were allowed to swim out freely into the buffer for fifteen minutes at room temperature. The tissue was then removed and sperm were either pelleted by centrifugation and stored at -80°C, or fixed to glass slides for immunocytochemical analysis. Sperm microsomes were prepared from frozen cell pellets as described for testis microsomes.

Aliquots of sperm were fixed to glass slides overnight in HistoPrep™ Buffered 10% Formalin (Fisher) at 4°C. The next morning, slides were washed in PBS and then used for immunocytochemical analysis. DAB detection of the α4 isoform in isolated sperm cells was performed from this point on as described above for the immunohistochemical analysis of testis sections. For immunofluorescent detection of the α4 isoform, slides were blocked for nonspecific binding by incubation in PBS containing 0.2% Triton X-100 and 2% normal donkey serum then placed in a solution containing αb4 (5.0 µg/ml). Immunofluorescent detection of the α1 isoform required exposure of cells to PBS containing 2% SDS for fifteen minutes, several washes in PBS and then incubation in solution containing α6f (1:100). On the third day, slides were washed (0.1 M PBS containing 0.2% Triton X-100) before incubation with the appropriate secondary antibody, either FITC-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) or Texas Red® dye-conjugated donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:100 for at least one hour. Slides were washed a final time and coverslips were mounted using Vectashield Mounting Medium for Fluorescence with DAPI (Vector Labs) for nuclei visualization. Fluorescently
labeled sperm cells were examined and photographed using the Axioplan 2 and Axiophot 2 light microscope equipped for fluorescence visualization by Zeiss (Thornwood, NY).

**Ouabain Binding Competition Assays** - All ouabain binding competition assays were performed as described previously (20,22). Three different sets of sperm microsomes from individual animals were analyzed to characterize each protein-ligand interaction. Purified sheep kidney enzyme was used as a positive control for individual assays. The $K_D$ for ouabain binding and IC$_{50}$ values for Na$^+$ and K$^+$ competition were determined as described previously, and errors reported are the standard errors from the mean for three samples.

**Sperm Motility Assays** – Sperm from whole epididymis were obtained as described above from Sprague-Dawley rats (23). A drop of the sperm suspension was then diluted in warmed, modified Tyrode's albumin lactate pyruvate (TALP) solution (24) (114 mM NaCl, 3.2 mM KCl, 2 mM NaHCO$_3$, 0.4 mM NaH$_2$PO$_4$H$_2$O, 10 mM Na lactate, 2 mM CaCl$_2$ 2H$_2$O, 0.5 mM MgCl$_2$ 6H$_2$O, 10 mM HEPES, 100 IU/ml Penicillin, 3 mg/ml BSA (fraction V) and 0.2 mM pyruvate). Ouabain solutions were prepared in TALPS media not more than 15 minutes before use and kept at 35°C. The percentage of motile sperm in the presence and absence of ouabain was determined using a hemocytometer to count duplicate samples in varied order. Motility was assessed every hour by counting at least 200 sperm per sample and sperm with any flagellar movement were scored as motile (25,26). The assay was performed on 5 different animals on separate days. The results from one day’s experiment, in duplicate, are shown and are representative of data collected in every experiment. Results are expressed as means ± standard error (SE) for duplicate samples from one animal on one day’s experiment. Statistical analyses of differences in sperm motility in ouabain compared to sperm motility in buffer alone were performed using unpaired Student’s $t$ test with equal variance.
RESULTS

Analysis of the α4 and α1 Isoforms in Maturing Testes - A variety of changes occur in the mammalian testis as it matures into a sexually active adult, including initiation of testosterone production, establishment of the blood-testis barrier and spermatogenesis (27,28). One of the first indications of sexual maturity is the production of mature sperm, called spermatozoa, in testis, an event that occurs in the rat at the average age of 33-35 days (29). Determination of the stage of sexual maturity during which the α4 isoform is produced will provide useful information concerning its functional role in testis. Therefore we began by examining its expression in both immature and mature animals, ages two to twelve weeks, at both the RNA and protein levels. Examination of total RNA isolated from testes from these animals revealed that the α4 isoform is not expressed until four weeks and reaches a maximum level, approximately three times the original amount, at six weeks (Figs. 1A and B). The ubiquitous α1 isoform, on the other hand, is expressed at a constant level throughout the life of the animal (Fig. 1A). Western analysis of the α4 isoform protein levels in these testes revealed that it is not present in testis until after four weeks, subsequent to which it increases almost three fold, while the α1 isoform is constantly present (Figs. 1C and D). The α4 isoform is therefore not omnipresent in testis, rather its expression is regulated in parallel to the onset of sexual maturity.

Localization of the α4 Isoform in Testes - Immunohistochemistry was next used to define the localization of expression of the α4 isoform in rat testis. Structurally, the testis divides into three regions, the membranous tunica albuginea that envelops the testis, the coiled seminiferous tubules, and the interstitium (28). The seminiferous tubules contain populations of both somatic cells, called Sertoli cells, and germ cells, which, beginning at puberty in mammals, develop and
mature through the process of spermatogenesis (27,28). Spermatogenesis begins with round, immature germ cells, and results in the production of elongated, mature sperm which are released into the luminal space (30). Intermediate forms of germ cells undergoing spermatogenesis include both round spermatocytes and elongating spermatids (30). Immunohistochemical examination of adult testis sections identified the α4 isoform in mature sperm, and no staining that indicates expression of the α4 isoform in any other cell type, including Sertoli cells and Leydig cells, was observed (Fig. 2A). In addition, immunoreactivity of the secondary antibody alone in testis was nonexistent (Fig. 2B).

The expression of the α4 isoform in mature sperm suggests that it must be present in germ cells in earlier stages of spermatogenesis, since mature sperm synthesize little to no new protein. Because spermatozoa represent a large population of cells in these adult testis sections, the strong α4 isoform-specific staining in these cells may mask the expression of this isoform in smaller populations of germ cells, such as spermatocytes and spermatids. Before the onset of spermatogenesis, spermatogonia are the only germ cells present in the mammalian testis (29,30). Testes of animals in which spermatogenesis has recently begun contain only early to intermediate developmental stages of germ cells with little to no spermatozoa. Investigation of testes from these animals therefore allows detection of the α4 isoform in developing germ cells in the relative absence of mature sperm. Towards this end, testis sections from animals three to twelve weeks old were examined for expression of the α4 isoform protein, and the presence of mature sperm was determined by hemotoxylin and eosin (H&E) staining. Testis sections from animals three and four weeks old do not express the α4 isoform protein (Fig. 2B), nor do they contain mature, elongating spermatozoa (Fig. 2B). Examination of testis sections from six week old animals, however, revealed some α4 isoform protein expression in intermediate stages of
A biological role for the Na,K-ATPase α4 isoform

developing sperm, possibly spermatids, and little to no spermatozoa present (Fig. 2B). Finally, testis sections from animals eight and twelve weeks old express the α4 isoform protein in spermatozoa (Fig. 2B), similar to the pattern described above for adult testes (Fig. 2A), and H&E stained tissues confirmed the abundance of mature sperm in the seminiferous tubule lumen (Fig. 2B). Again, testis sections incubated with the secondary antibody alone did not show any immunoreactivity (Fig. 2B). The expression of the α4 isoform protein therefore immediately follows the onset of spermatogenesis, and its expression is localized to mature spermatozoa and some intermediate stages of developing sperm.

Identification of Na,K-ATPase α Isoforms in Sperm - Microsomes of isolated sperm collected from the epididymis of sexually mature rats were subsequently examined for expression of each of the four α isoforms of the Na,K-ATPase. Western blots containing microsome samples from testis, sperm, red blood cells and brain were probed individually using α isoform-specific antibodies. These westerns revealed that sperm express only the α4 and α1 isoforms, and compared to testis the level of expression of the α4 isoform is very high while that of the α1 isoform is low (Fig. 3). As expected, whole testis expresses the α4 and α1 isoforms, red blood cells, included since they are the only cell contaminant in sperm preparations, express only the α1 isoform, and brain expresses the α1, α2 and α3 isoforms (Fig. 3).

Localization of the α4 and α1 Isoforms in Sperm - The distributions of the α4 and α1 isoforms of the Na,K-ATPase were next examined in isolated sperm cells. Immunocytochemical localization of the α4 isoform by diaminobenzidine (DAB) staining identified the α4 isoform specifically in the flagellum of the sperm, most heavily perceived in the midpiece region of the tail (Fig. 4A). Sperm incubated without primary antibody did not show any staining (Figs. 4B). Immunofluorescent labeling of the α4 isoform in sperm confirmed this pattern of expression.
A biological role for the Na,K-ATPase α4 isoform

(Figs. 4C and D). The entire sperm flagellum was examined for α4 isoform expression by sequentially photographing sperm with first the head then the tail in the focal plane (Figs. 4C and D). This paired series of photographs showed that regardless of the focal plane, the expression pattern of the α4 isoform protein is localized to the midpiece of the flagellum and there is no detectable secondary antibody immunoreactivity (Figs. 4C, D, F, and G). Visualization of the α1 isoform revealed its location in the same region of the sperm where the α4 isoform was found, while nonspecific secondary antibody binding to sperm was undetectable (Fig. 4, E and H). Therefore, both of the α isoforms of the Na,K-ATPase present in sperm are localized to the midpiece of the flagellum and do not have distinct patterns of localization that are detectable at this level of resolution.

**Biochemical Analysis of the α4 Isoform in Sperm** - The biochemical characteristics of the α4 isoform in sperm were next examined in order to define any distinguishing characteristics between it and the α1 isoform. Our laboratory and others have previously measured the biochemical characteristics of the α4 isoform using tissue culture cell expression systems (20,21), but it is important to examine this isoform in endogenous cells as differences in kinetics of the Na,K-ATPase are not only isoform-specific, but are also tissue-specific (31,32). Using ouabain binding competition assays (20,22), the ligand binding affinities for the α4 isoform in sperm microsomes were found to be similar to those previously reported (20), and are listed here as mean values ± the standard error between three unique samples: the K_D for ouabain binding is 148.50 ± 23.44 nM, the IC_{50}(Na^+) is 6.13 ± 0.62 mM, and the IC_{50}(K^+) is 3.49 ± 0.35 mM.

**Ouabain Inhibition of Sperm Motility and Fertilization** - The results presented in this paper thus far have defined the α4 isoform of the Na,K-ATPase to be specific to sperm. The identification of the α4 isoform in spermatozoa and not in spermatogonia suggests that it’s biological role must
be related to a specialized function of mature sperm, for example, flagellar movement for sperm motility. The Na,K-ATPase is the molecular receptor for cardiac glycosides such as ouabain, which bind to and inhibit the activity of the enzyme (17). The effects of ouabain on sperm motility have been previously examined in other species, but the results were interpreted without considering the existence of Na,K-ATPase molecules carrying different α isoforms (33). Two α isoforms of the Na,K-ATPase have now been identified in rat sperm: the high affinity ouabain receptor, α4, and the low affinity ouabain receptor, α1. Because of their different pharmacological properties, the effects of ouabain inhibition of the α4 isoform alone on sperm motility were examined. Freshly isolated epididymal rat sperm were incubated in buffer containing either 1x10^{-5} M ouabain, which will only inhibit the α4 isoform, or 1x10^{-2} M ouabain, which will inhibit both the α4 and α1 isoforms. The percentage of motile sperm in each ouabain solution was counted each hour and compared to the percentage of motile sperm in buffer alone (Fig. 5). The results from these motility assays revealed that ouabain inhibition of the α4 isoform alone is sufficient to reduce sperm motility to the same level as ouabain inhibition of all of the Na,K-ATPase, while the motility of sperm in control buffer is constant and sometimes even increases over the time span of each experiment, up to eighteen hours (Fig. 5 and data not shown). Motility was assessed by scoring sperm with any flagellar movement as being motile. The residual motile sperm observed in ouabain solutions exhibited little to no forward movement and overall less activity compared to the motile sperm in control buffer, indicating that sperm movement is essentially abolished by inhibiting α4. These data clearly show the dependence of sperm motility on the α4 isoform, leading to the question of the consequences of α4 inhibition on fertilization. Interestingly, the effect of ouabain inhibition of the Na,K-ATPase on in vitro fertilization (IVF) has been previously examined in the mouse but again these results were
interpreted without considering the presence of multiple $\alpha$ isoforms in sperm (34). In that study, acrosome-reacted spermatozoa were exposed to different concentrations of ouabain and their ability to successfully fertilize zona-free mouse oocytes was examined (34). Compared to control studies in the absence of ouabain, the number of oocyte fertilizations by sperm exposed to low concentrations of ouabain ($1 \times 10^{-5}$ M) was maximally reduced to 0-5% (34). Ongoing studies from our laboratory have defined the $\alpha$ isoforms of the Na,K-ATPase in mouse testis to be identical to those in rat (4, manuscript in preparation). Therefore, the inhibition of fertilization events observed at low concentrations of ouabain in the mouse can only be attributed to specific inhibition of the $\alpha 4$ isoform in sperm, revealing a critical role for this isoform in both sperm motility and fertilization.

DISCUSSION

One of the major objectives of our laboratory is to identify and define specific functional roles for $\alpha$ isoforms of the Na,K-ATPase. Until now, a unique role has only been demonstrated for the $\alpha 2$ isoform as a $Ca^{2+}$ regulator in cardiac myocytes (17). The data presented here constitute the first description of a biological function for the most recently characterized Na,K-ATPase $\alpha$ isoform, $\alpha 4$. Expression of the $\alpha 4$ isoform has now been identified in spermatozoa, specifically in the midpiece of the flagellum, suggesting a functional role related to the specialized activity of these cells. In fact, this novel Na,K-ATPase isoform does play a critical role in sperm function since selective inhibition of the $\alpha 4$ isoform alone is sufficient to eliminate sperm motility, providing new perspectives in both the studies of biological functions of Na,K-ATPase $\alpha$ isoforms and of general mechanisms of sperm motility.
Sperm motility is dependent on a number of different parameters, one of which is the cytosolic pH (35,36). Environmental conditions that inhibit sperm motility, such as the absence of Na\(^+\), also decrease the intracellular pH, resulting in a more acidic cytoplasm in these immobile sperm than in mobile sperm (35,36). The reinitiation of motility of these sperm, by resuspension in Na\(^+\) containing media, is immediately preceded by their release of H\(^+\) (35). In fact, the addition of NH\(_4\)Cl (35) or bicarbonate (36) alone to the external media, both of which stimulate H\(^+\) release, is also sufficient to induce sperm motility. These findings highlight the importance of H\(^+\) extrusion and the regulation of intracellular pH for the initiation and maintenance of sperm motility.

The Na/H exchangers are a family of proteins involved in intracellular pH regulation in many epithelial tissues (37) and recently, the Na/H exchanger-1 (NHE-1) protein has been detected in porcine spermatozoa (36). The Na,K-ATPase establishes the Na\(^+\) gradient across the membrane which the Na/H exchanger uses to remove H\(^+\) from the cell (37). The functional role of the Na/H exchanger in regulating the internal pH of sperm has been investigated using ouabain (35), a specific inhibitor of the Na,K-ATPase, amiloride (35), an inhibitor of the Na/H exchanger, and the amiloride analog, 5-((N-ethyl-N-isopropyl) amiloride (EIPA) (36). These drugs were shown to inhibit acid release, internal pH recovery, and motility initiation, and together with the identification of NHE-1 in sperm, suggest that proper functioning of the Na/H exchanger is essential for regulating intracellular pH, and therefore motility of sperm (35,36). Specific inhibition of the Na,K-ATPase carrying the \(\alpha4\) isoform likely induces intracellular acidification of sperm by eliminating Na\(^+\) gradients necessary for the Na/H-exchanger to remove excess H\(^+\) resulting in the loss of motility (Fig. 6). The localization of the Na,K-ATPase to the midpiece region of the sperm where the mitochondria are found (38) is therefore not surprising.
since sperm mitochondria are responsible for producing the ATP necessary for flagellar movement, and during this metabolic activity large amounts of H\(^+\) leak from the mitochondrial inner membrane space into the cytoplasm (39). In the sperm midpiece, the mitochondria lies directly below, and possibly in contact with the plasma membrane (38), providing the likelihood for the existence of a restricted-volume space in these cells into which H\(^+\) leaks from the mitochondria. The presence of a unique isoform of the Na,K-ATPase, working in concert with the Na/H exchanger in the midpiece, would thereby provide a mechanism for the tight control of H\(^+\) concentration in this region of the sperm allowing for normal sperm motility (Fig. 6).

The acidic internal environment in sperm may directly affect flagellar movement through inhibition of dynein activity. Structurally, the sperm tail is a flagellum, a cellular component comprised of microtubules whose movement is powered by the ATPase motor, dynein (30). In flagella, the outer and inner dynein arms are the axonemal structures involved in the production of the stroke necessary for the sliding of adjacent microtubules (40). Recently, one study demonstrated that sperm lacking outer dynein arms do not increase motility in response to an increase in pH, which stimulates normal sperm, but produce a low level of constant motility at an acidic pH where normal sperm are relatively inactive. These data suggest that the outer dynein arms contain a pH sensitive regulatory mechanism (40). Therefore, in an acidic environment, dynein outer arms may inhibit flagellar activity, while in more alkaline conditions, dynein outer arms activate flagellar activity and motility.

The mechanism of reducing sperm motility by specifically inhibiting the \(\alpha_4\) isoform may also involve disruption of the plasma membrane potential. The plasma membrane potential of a spermatozoa undergoes many changes throughout maturation that are critical for it’s ability to fertilize the ovum, therefore precise regulation of the plasma membrane potential is necessary for
normal sperm function (41). Previous studies, using bull sperm, have shown that ouabain decreases the progressive motility and flagellar wave of sperm and that the membrane potential of these sperm is dramatically more positive, providing a connection between the regulation of membrane potential and sperm motility (42). The contribution of individual Na,K-ATPase α isoforms to this phenomenon was not considered. The inhibition of sperm motility by the loss of the α4 isoform alone may therefore be a result of disturbing the regulation of the membrane potential.

Interestingly, a recent study of immotile sperm collected from asthenozoospermic, infertile humans, demonstrated that they exhibit both decreased motility and dramatically less negative plasma membrane potentials compared to sperm from healthy, normal, fertile men (41). Again, the contribution of individual Na,K-ATPase α isoforms was not considered, but future studies of these infertile patients may reveal the absence of, or dysfunctional, Na,K-ATPase carrying the α4 isoform. Once this has been established, agents that increase Na,K-ATPase activity or gene therapy techniques that introduce a functional α4 isoform gene into sperm could be used to restore motility and fertility in these patients.

Another application suggested by the data presented here on the α4 isoform of the Na,K-ATPase involves the design of unique pharmacological agents that exclusively inhibit the α4 isoform for use as male birth control agents. The protein sequence of the α4 isoform is the least similar of the four α isoforms of the Na,K-ATPase (13) which should facilitate the design of specific inhibitors. In addition, the limited expression pattern of the α4 isoform suggests that patients treated with these inhibitors would only see effects on sperm without consequence to other organ functions.
The localization of the α4 isoform in sperm cells and identification of its critical role in sperm motility and fertilization now necessitate further study of its contribution to other sperm-specific biochemical processes, including capacitation and the acrosome reaction (43-46). These studies will provide a better understanding of the spectrum of biological functions connected to this novel isoform of the Na,K-ATPase and the potential use of this protein as a specific target for male fertility/contraception treatments.

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A biological role for the Na,K-ATPase α4 isoform

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FIGURE LEGENDS

Fig. 1. **The Na,K-ATPase α4 and α1 isoforms exhibit different expression patterns in testis throughout sexual maturation.** Northern blot and western blot analyses of the expression of the α4 and α1 isoforms in testes from rats between the ages of 2 and 12 weeks. *A*, Northern blots probed sequentially for the expression of the α4 and α1 isoforms showed that the α4 isoform is not expressed until 4 weeks, while the α1 isoform is expressed constantly throughout the life of the animal. GAPDH expression was used as a loading control. *B*, Quantitation of the levels of α4 isoform expression, normalized to GAPDH expression, revealed an approximately three fold increase in α4 isoform expression after 4 weeks. *C*, Western blots showed that the α4 isoform protein is not present in testis until after 4 weeks, while the α1 isoform protein is present at constant levels throughout sexual maturation. 20 µg of protein is loaded in each lane. *D*, Quantitation of the α4 isoform protein levels revealed an approximately three fold increase in α4 isoform expression after 6 weeks.
A biological role for the Na,K-ATPase α4 isoform

Fig. 2. Immunohistochemical analyses revealed the α4 isoform is localized to spermatozoa.

A, An adult testis section incubated with the α4 isoform-specific antibody showed the presence of the α4 isoform in mature sperm localized to the center of the seminiferous tubule, while sections incubated with the secondary antibody alone showed no cross-reactivity. Bar = 65 µm.

B, Testes sections from animals 3 to 12 weeks old were probed for expression of the α4 isoform protein, exposed to the secondary antibody alone, and stained with hematoxylin and eosin (H&E). Only testis sections containing mature sperm cells showed specific staining for the α4 isoform protein. Testes sections incubated in secondary antibody alone showed no cross reactivity. Hematoxylin and eosin staining revealed mature sperm in the lumen of seminiferous tubules from animals ages 6, 8 and 12 weeks old and not in the seminiferous tubules of younger animals 3 and 4 weeks old. Bar = 75 µm.

Fig. 3. The α4 and α1 isoforms of the Na,K-ATPase are expressed in rat epididymal sperm.

The expression of each of the α isoforms of the Na,K-ATPase was examined in testis, sperm, red blood cells and brain microsomes by western blot analysis. 10 µg of protein was loaded in each lane, except the lane for the brain sample which contained 1 µg of protein. The α4 isoform is only in testis and sperm microsomes, the α1 isoform is in all samples, and the α2 and α3 isoforms are only in brain microsomes.

Fig. 4. The α4 and α1 isoforms of the Na,K-ATPase are localized to the midpiece region of sperm. A, Diaminobenzidine staining of isolated sperm cells incubated with an α4 isoform-specific antibody revealed high expression of this isoform in the midpiece of the flagellum, B, while sperm cells incubated with the secondary antibody alone revealed no cross reactivity.
Black bars = 25 µm. C, A sperm cell incubated in both an α4 isoform-specific antibody and a FITC-conjugated secondary antibody, taken with the head of the sperm cell in the focal plane, revealed intense α4 isoform protein expression in the midpiece of the flagellum. D, The same sperm cell was photographed with the end of the flagellum in the focal plane, again revealed intense α4 isoform protein expression only in the midpiece of the flagellum. E, A sperm cell incubated in both an α1 isoform-specific antibody and a Texas-Red-conjugated secondary antibody, taken with the head of the sperm in the focal plane, revealed α1 isoform protein expression in the head and midpiece of the flagellum. F-H, Isolated sperm cells incubated in either the FITC conjugated- or Texas Red-conjugated secondary antibody alone did not show any crossreactivity. White bars = 20 µm.

Fig. 5. Inhibition of sperm motility by low doses of ouabain. The percentage of motile sperm in TALPS solution containing 0 M, 1x10⁻⁵ M or 1x10⁻² M ouabain was reported every hour, and revealed a significant inhibition of motility for sperm incubated in both concentrations of ouabain. The percentage of motile sperm incubated in 1x10⁻² M ouabain was never statistically less than that of sperm in 1x10⁻⁵ M ouabain, showing that inhibition of the α4 isoform alone is sufficient to eliminate sperm motility. In addition, motile sperm in ouabain solutions exhibited little to no forward movement compared to motile sperm in TALPS alone. \( P \) values <0.05 are indicated by * , \( P \) values <0.001 are indicated by ** above each bar.

Fig. 6. Model for the Na,K-ATPase α4 isoform’s functional role in sperm motility. The H⁺ leak from the mitochondria during chemiosmotic synthesis of ATP is regulated through the enzymatic activity of the Na,K-ATPase working in concert with the Na/H exchanger.
Abbreviations used: $\alpha_4$, Na,K-ATPase $\alpha_4$ isoform; $\alpha_1$, Na,K-ATPase $\alpha_1$ isoform; NHE, Na/H exchanger.
Sperm motility is dependent on a unique isoform of the Na,K-ATPase
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