Activity of the Na,K-ATPase α4 isoform is important for membrane potential, intracellular Ca^{2+}, and pH to maintain motility in rat spermatozoa

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Abstract

While the function of the ubiquitous Na,K-ATPase α1 subunit has been well documented, the role of the sperm-specific α4 isoform of this ion transporter is less known. We have explored the importance of α4 in rat sperm physiology by taking advantage of the high sensitivity of this isoform for the inhibitor ouabain. Using concentrations that selectively block α4 activity, we found ouabain to reduce not only sperm total motility, but also multiple parameters of sperm movement, including progressive motility, straight line, curvilinear, and average path velocities, lateral head displacement, beat cross frequency, and linearity. According to a direct role of α4 in Na^{+} transport, ouabain inhibition of α4 increased [Na^{+}], in the male gametes. In addition, interference of α4 activity with ouabain produced cell membrane depolarization, diminished pH, and increased [Ca^{2+}], in spermatozoa. Inhibition of α4 was sufficient to cause all these effects and additional blockage of α1, the other Na,K-ATPase α isoform expressed in sperm, and higher doses of ouabain did not result in further changes in the cell parameters studied. These results show that α4 is the Na,K-ATPase isoform primarily involved in controlling the transmembrane Na^{+} gradient in sperm, and that α4 activity is necessary for maintaining membrane potential, [Ca^{2+}]/i, and [H^{+}], in the cells. The high dependence of sperm motility on membrane excitability, [Ca^{2+}]/i, and acid–base balance suggests that their regulation is the mechanism by which α4 maintains motility of the male gametes.


Introduction

The Na,K-ATPase or Na pump is an enzyme of the plasma membrane of most cells that transduces the energy from the hydrolysis of ATP to catalyze the exchange of cytoplasmic Na^{+} for extracellular K^{+} (Kaplan 2002). In somatic cells, the ion gradients generated by the Na,K-ATPase play a central role in maintaining cell volume and pH, in keeping cell resting membrane potential, and in providing the chemical energy necessary for the secondary transport of other ions, solutes, and water across the cell surface (Hoffmann & Simonsen 1989, Gloor 1997, Feraille & Doucet 2001). The Na,K-ATPase is an oligomer composed of two major polypeptides, the α and β subunits (Morth et al. 2007). The α polypeptide constitutes the catalytic subunit of the Na,K-ATPase, which directly participates in the ion translocation and hydrolytic activity of the enzyme. It contains the binding sites for Na^{+}, K^{+}, and ATP, and for the cardenolide, ouabain, which is a potent inhibitor of the Na,K-ATPase (Kaplan 2002).

Interestingly, four structural variants or isoforms of the Na,K-ATPase α subunit, namely α1, α2, α3, and α4, are expressed in mammalian tissues. Each of these isoforms is characterized by a cell-specific pattern of expression, and by particular enzymatic properties (Blanco 2005a, 2005b). While α1 is found in nearly every tissue and it is believed to maintain the basal ion gradients in the cells, the other α polypeptides are more restricted in their expression and appear to play tissue-specific roles (Blanco & Mercer 1998, Mobasher et al. 2000). In particular, the α4 isoform is found in the testis, where it is specifically expressed in the male germ cells after meiosis (Shamraj & Lingrel 1994, Woo et al. 1999, 2000, Blanco et al. 2000, Hlivko et al. 2006). The α4 isoform is highly prevalent in spermatozoa, and its activity corresponds to approximately two-thirds of the total Na^{+} and K^{+} active transport of the cells (Wagoner et al. 2005). The only other Na,K-ATPase α polypeptide present in sperm is the widely expressed α1 isoform (Wagoner et al. 2005). We have previously shown that the activity of α4 is different from that of α1 and the other Na,K-ATPase α isoforms. Thus, α4 exhibits unique...
kinetic properties for its physiological ligands, including a high affinity for $\text{Na}^+$, a low affinity for $\text{K}^+$, and an intermediate affinity for ATP (Blanco et al. 1999, Sanchez et al. 2006). Moreover, $\alpha 4$ has a distinctive very high sensitivity to ouabain, a characteristic that most prominently distinguishes it from the $\alpha 1$ isoform (Blanco et al. 1999, Sanchez et al. 2006). The presence of a Na,K-ATPase catalytic subunit with particular attributes, such as those of $\alpha 4$, suggests that this polypeptide plays a specific role in the physiology of sperm. In support of this, inhibition of $\alpha 4$ activity has been shown to diminish total motility of spermatozoa (Woo et al. 2000, Sanchez et al. 2006).

Despite the progress made in understanding various characteristics of the expression and activity of $\alpha 4$, its role in different aspects of sperm motility and the mechanisms by which this isoform supports sperm physiology remain unknown. Deciphering the molecular basis of $\alpha 4$ action is an important step in comprehending how this isoform contributes to male fertility. In the present work, we have studied the role of $\alpha 4$ in different parameters of sperm movement and in a series of processes that are essential for sperm motility. For this, we have taken advantage of the distinctive high sensitivity of $\alpha 4$ for ouabain to specifically inhibit this isoform, and to distinguish its activity from that of the $\alpha 1$ isoform in rat spermatozoa. Our results show that $\alpha 4$, but not the $\alpha 1$ isoform, is important in maintaining sperm intracellular $\text{Na}^+$ concentration ([Na$^+$]), membrane potential, intracellular pH, and cytoplasmic $\text{Ca}^{2+}$ concentration ([Ca$^{2+}$]). The control of these cell parameters is essential for sustaining sperm motility (Quill et al. 2006, Darszon et al. 2007), suggesting that their regulation by $\alpha 4$ represents the mechanism through which this Na,K-ATPase isoform supports motility of the male gametes.

**Results**

**Inhibition of Na,K-ATPase $\alpha 4$ isoform inhibits multiple parameters of rat sperm motility**

We and others have shown previously that activity of the Na,K-ATPase $\alpha 4$ isoform is important for motility of rat and human sperm (Woo et al. 2000, Sanchez et al. 2006). However, previous experiments have only focused on the general movement of the spermatozoa, and observations were based on bare visual determinations (Woo et al. 2000, 2002). To further study the relevance of $\alpha 4$ to not only total motility, but also other parameters of sperm movement, we determined the effects of inhibition of this isoform on sperm motility using computer-assisted sperm analysis (CASA). We also assessed the relative contribution of $\alpha 4$ to sperm motility, and compared it with that of the $\alpha 1$ isoform. Extensive studies of the kinetic properties of the Na,K-ATPase showed that each of the Na,K-ATPase isoforms expressed in sperm, $\alpha 1$ and $\alpha 4$, exhibits a marked difference in affinity for the inhibitor ouabain (Blanco 2005a). In rats, the ouabain affinity of $\alpha 4$ is $\sim 10 000$-fold higher than that of $\alpha 1$ (Wagoner et al. 2005). In this manner, differential sensitivity to ouabain has been used as a tool to specifically inhibit $\alpha 4$ activity in a dose-selective manner to study the particular function of this isoform in spermatozoa (Blanco 2005a). Thus, as we have reported previously, ouabain at a concentration of $10^{-6} \text{M}$ is sufficient to completely inhibit $\alpha 4$ activity, without having significant effects on the $\alpha 1$ polypeptide. A ouabain concentration of $10^{-3} \text{M}$ instead will cause inactivation of both the $\alpha 1$ and $\alpha 4$ isoforms (Wagoner et al. 2005). Based on this, we treated rat spermatozoa in the absence and presence of $10^{-6}$ and $10^{-3} \text{M}$ ouabain for different times, and measured the motility of the cells using CASA. As shown in Fig. 1A, in a medium without ouabain, rat sperm total motility diminished

![Figure 1](image-url)
only slightly during the entire incubation period of 2 h. In contrast, ouabain inhibition of $\alpha 4$ was sufficient to impair the percent of total motile spermatozoa in a time-dependent manner. Further inhibition of $\alpha 1$ with $10^{-3}$ M ouabain did not result in additional reduction of sperm motility. Ouabain at $10^{-6}$ M, similar to that at $10^{-3}$ M, also diminished rat sperm progressive motility (Fig. 1B). In addition, ouabain-selective blockage of $\alpha 4$ activity affected other parameters of sperm motility, including straight line, curvilinear, and average path velocities, amplitude of lateral head displacement, beat cross frequency, and linearity (Fig. 2A–F). Altogether, these results suggest that the activity of the Na,K-ATPase is important for sustaining multiple aspects of sperm flagellar movement, and that the $\alpha 4$ isoform, and not $\alpha 1$, is involved in this process.

**Inhibition of $\alpha 4$ activity affects intracellular Na$^+$ in rat spermatozoa**

The low cytoplasmic concentration of Na$^+$ that typically exists in most cells is a direct consequence of the active ion transport function of the Na,K-ATPase (Kaplan 2002). This Na$^+$ gradient in turn is essential for many general and cell-specific processes (Feraille & Doucet 2001, Kaplan 2002). We have demonstrated previously that the $\alpha 4$ isoform functions as an active Na,K-ATPase, catalyzing the ouabain-dependent hydrolysis of ATP in rat sperm (Wagoner et al. 2005). However, the extent to which the activity of $\alpha 4$ as an ion transporter contributes to maintain sperm [Na$^+$], is unknown. To specifically determine the role of $\alpha 4$ in [Na$^+$], maintenance and to correlate its transport activity with that of the $\alpha 1$ isoform, we treated rat spermatozoa without and with $10^{-6}$ and $10^{-3}$ M ouabain, and we measured Na$^+$ in the cells using the fluorescent indicator, sodium green tetraacetate. Figure 3A shows a representative trace corresponding to the fluorescence recorded in sperm without or with the indicated concentrations of ouabain. Figure 3B summarizes the compiled values for the intensity of fluorescence in the cells from different samples, expressed relative to the untreated controls. As expected, blockage of the Na,K-ATPase with ouabain, even after a relatively short time of incubation with the inhibitor (30 min), caused [Na$^+$], to increase in sperm by $\sim 20\%$. Interestingly, the major changes in [Na$^+$] were observed after inhibition of the $\alpha 4$ isoform with $10^{-6}$ M ouabain, and no further significant increase in [Na$^+$] was detected after inhibition of the Na,K-ATPase $\alpha 1$ polypeptide with $10^{-3}$ M ouabain. These results suggest that the activity of the $\alpha 4$ isoform exerts a more prominent effect in maintaining the [Na$^+$] in spermatozoa (Wagoner et al. 2005).

**Function of $\alpha 4$ is important for sperm membrane potential**

The transmembrane distribution of electrical charges, resulting from the uneven Na$^+$ and K$^+$ transport activity of the Na,K-ATPase, contributes to maintain the resting membrane potential that provides cells with the excitability required for their movement.

**Figure 2** Activity of the Na,K-ATPase $\alpha 4$ isoform is important for several parameters of rat sperm motility. Rat spermatozoa from the cauda of epididymides were isolated and treated with modified Tyrode’s medium in the absence and presence of $10^{-6}$ and $10^{-3}$ M ouabain. Sperm motility was determined using CASA, and different parameters were analyzed after 1 h of treatment without and with the indicated amounts of ouabain using the SpermVision system. (A) Straight line velocity, VSL; (B) average path velocity, VAP; (C) curvilinear velocity, VCL; (D) amplitude of lateral head displacement, ALH; (E) beat cross frequency, BCF; and (F) linearity. Cells from ten different fields per condition were analyzed. Bars represent the mean $\pm$ S.E.M. of the mean of ten experiments. Bars represent untreated cells (open bars), or cells treated with $10^{-6}$ M ouabain (gray bars) and $10^{-3}$ M ouabain (black bars). Values significantly different from the control are indicated with an asterisk, with $P<0.001$. No statistical differences were found between samples treated with $10^{-6}$ and $10^{-3}$ M ouabain.
absence and presence of 10 potential. For this, we treated rat spermatozoa in the
with P significantly different from the control are indicated with an asterisk,
10 [Na]
indicator sodium green tetraacetate was used to follow changes in
differences were found between samples treated with 10 with 10
collected and incubated in modified Tyrode’s medium without and
particular that of the Na,K-ATPase, and in
(Hernandez-Gonzalez et al. 2006). Figure 4A presents
contraction, and transmission of impulses (Gloor 1997).
We investigated the role of the Na,K-ATPase, and in particular that of the z4 isoform, in sperm membrane
potential. For this, we treated rat spermatozoa in the
absence and presence of 10−6 and 10−3 M ouabain, and determined membrane potential of the cells using the fluorescent indicator [DiSC3(5)] as described (Hernandez-Gonzalez et al. 2006). Figure 4A presents representative traces for the fluorescence measured in untreated and ouabain-treated cells. As shown, the initial portion of the recordings represents the fluorescence emission corresponding to the membrane potential of the cells in the absence or presence of the indicated ouabain concentrations. Each determination was followed by a calibration curve, which was obtained in the same sample after the addition of the ionophore valinomycin and increasing amounts of KCl. As reflected in the traces, valinomycin produced K+ leakage from the cells and membrane hyperpolarization, while the subsequent addition of KCl caused a stepwise depolarization of the cells. This calibration method allowed calculation of the cell membrane potential of the samples in the absence and presence of ouabain (Hernandez-Gonzalez et al. 2006). Figure 4B illustrates the values of membrane potential for each experimental condition, averaged from different experiments. As shown, 10−6 M ouabain produced depolarization of the cells, with a decrease in sperm membrane potential of ~20 mV. A similar reduction in membrane potential
was obtained with 10−3 M ouabain. This suggests that the Na,K-ATPase importantly contributes to maintain membrane potential in rat spermatozoa, and that α4 is the isoform primarily involved in this process.

The α4 isoform contributes to pH balance in spermatozoa
Regulation of intracellular pH is essential for the function of sperm (Darszon et al. 1999). An important mechanism that controls proton concentration in somatic cells operates utilizing the transmembrane Na+ gradient generated by the Na,K-ATPase. In this manner, the Na,K-ATPase indirectly controls pH in the cells (Bers et al. 2003, Despa et al. 2004). We explored whether the Na,K-ATPase plays a similar role in spermatozoa. Moreover, we determined whether α4 or the ubiquitous α1 isoform is involved in the secondary regulation of H+ content in the male gametes. For this, rat spermatozoa in modified Tyrode’s medium were treated without and with 10−6 and 10−3 M ouabain, and changes in intracellular pH were followed using the fluorescent indicator, SNARF-1. In order to study ouabain effects at a range of physiological pH values, experiments were performed by incubating the cells in the media with pHs of 7.0, 7.2, 7.4, and 7.6. Under these conditions, sperm viability, measured using the fluorescent marker, dye cycle violet, was 99 ±1% (data not shown). Figure 5A shows a representative trace carried out at an extracellular pH of 7.4. The initial segments in this trace correspond to samples treated without and with the indicated ouabain concentrations, followed by recordings of samples clamped at preset intracellular pH values, which served for calibration (Chow & Hedley 2007). Figure 5B depicts the compiled data for controls and ouabain-treated samples from a series of experiments performed at the indicated extracellular pHs. As shown, the intracellular pH of sperm rose after incubation of the cells in the media with increasing pHs. Importantly, under the various pHs tested, ouabain caused acidification of the cells. In addition, in all cases, the primary decrease in pH occurred after applying 10−6 M ouabain to the cells, with no significant changes in proton levels after further treatment with 10−3 M ouabain. This suggests that the Na,K-ATPase is involved in the acid–base balance in spermatozoa, and that the activity of the α4 isoform plays a fundamental role in maintaining proton concentration low in the cells.

Activity of α4 plays a role in sperm intracellular calcium maintenance
The Na+ gradient generated by the Na,K-ATPase is also important for the regulation of Ca2+ levels in some cell types, such as muscle and cardiac cells. To explore if, in spermatozoa, [Ca2+]i, is regulated through the Na,K-ATPase and to ascertain the relative involvement
of the α1 and α4 isoforms of the transporter in this event, we determined Ca\(^{2+}\) concentration in rat sperm at the single cell level using the fluorescent dye, calcium green. This was performed after treatment of the cells in the absence and presence of 10\(^{-6}\) and 10\(^{-3}\) M ouabain in Tyrode's modified medium. As shown in Fig. 6A, ouabain treatment resulted in significant increases in the calcium green signal in the cells, indicating a rise in [Ca\(^{2+}\)], in spermatozoa. In addition, ouabain inhibition of α4 was sufficient to cause the maximal ouabain-dependent augment of [Ca\(^{2+}\)], in the male gametes. The use of 10\(^{-3}\) M ouabain did not produce further changes in [Ca\(^{2+}\)]. Figure 6B shows the quantification of the averaged data for [Ca\(^{2+}\)], for the control and ouabain-treated spermatozoa from various experiments. As shown, 10\(^{-6}\) and 10\(^{-3}\) M ouabain caused an increase of ~60% in sperm [Ca\(^{2+}\)]. These data suggest that the Na,K-ATPase, and more specifically its α4 isoform, controls sperm [Ca\(^{2+}\)].

**Discussion**

The maintenance of differences in ion concentration between the environment and the sperm cytoplasm has long been recognized as essential for normal motility of the male gametes (Darszon et al. 1999, 2006). Of particular importance is the uneven transmembrane distribution of Na\(^{+}\) and K\(^{+}\) generated by the Na,K-ATPase (Darszon et al. 1999). However, the specific contribution of different Na,K-ATPase isoforms in sperm physiology is not precisely known. In this work, we have determined the role of the Na,K-ATPase α4 isoform in various aspects of the motility of rat sperm, and have explored the mechanisms by which this transporter functions in the male gametes. Because animal models in which the α4 isoform has been deleted are not yet available, our study has taken advantage of the unique high affinity of α4 to ouabain to specifically inhibit this isoform (Woo et al. 2000, Wagoner et al. 2005). This method provides a suitable pharmacological approach, and is currently the best tool available to study the function of the α4 isoform in the native spermatozoa. We have shown that inhibition of α4, with relatively low amounts of ouabain, significantly affects multiple aspects of sperm motility. The use of CASA has allowed us to establish in more detail the role of α4 in different components of rat sperm movement, beyond the resolution given by previous simple visual determinations. Thus, we found that activity of α4 is necessary for sperm total and progressive motility, straight line, curvilinear, and average path velocities, lateral head displacement, beat cross frequency, and linearity. The alterations in the parameters of sperm motility that we have studied have been used as important predictors of male fertility in patients and for IVF purposes (Moore & Akhondi 1996, Larsen et al. 2000, Shibahara et al. 2004). Based on these reports, the extent of the reduction in the various parameters of sperm movement that we obtained after treatment with 10\(^{-6}\) M ouabain is expected to interfere with normal male fertility (Moore & Akhondi 1996, Larsen et al. 2000, Shibahara et al. 2004). Therefore, our results indicate that activity

**Figure 4** Na,K-ATPase α4 isoform activity contributes to maintain rat sperm membrane potential. Rat spermatozoa from the cauda epididymides were isolated and incubated for 30 min in modified Tyrode's medium in the absence and presence of 10\(^{-6}\) and 10\(^{-3}\) M ouabain. Then, membrane potential of the cells was determined using the fluorescent indicator [DSC(+)5] as described in Materials and Methods. (A) Representative traces for the control and ouabain-treated cells, which were obtained at an excitation/emission wavelength of 620/670. Calibration was done 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 KCl, and the subsequent KCl additions (indicated with the letters a–d) resulted in the following final KCl concentrations in millimolar: a) 9.9, b) 13.9, c) 21.9, and d) 37.9, which corresponded to the indicated membrane potentials (ΔEm) in millivolts respectively. (B) Compiled data from three different experiments each performed in triplicate. Bars represent the mean ± S.E.M. Values significantly different from the untreated control are indicated with an asterisk, with P<0.03 for 10\(^{-6}\) M ouabain versus untreated control and P<0.04 for 10\(^{-3}\) M ouabain versus untreated control. No statistical differences were found between samples containing 10\(^{-6}\) and 10\(^{-3}\) M ouabain (P<0.9).
of the Na,K-ATPase α4 polypeptide widely influences sperm movement, and suggest that this isoform is important to support motility and fertility of the male gametes. The broad effect of inhibition of α4 activity on the various components of sperm motility suggests that this isoform maintains sperm movement by acting through multiple different vital parameters of sperm physiology.

We have found that the α4 isoform is primarily involved in maintaining the low [Na+]i levels in rat spermatozoa. This agrees with our previous findings showing that α4 is responsible for most of the Na,K-ATPase catalytic activity of the cells (Wagoner et al. 2005). The importance of the α4 isoform as the main transport system for Na⁺ and K⁺ in spermatozoa is also evident from our observation that 10⁻⁶ M ouabain caused maximal depolarization of the sperm plasma membrane. The Na,K-ATPase itself is not the only determinant of membrane potential, and the simultaneous ‘leak’ of K⁺ via K⁺ channels is the other necessary component for the maintenance of membrane electrical polarity in the cells (Green 2004). Therefore, it is obvious that for the α4 isoform to influence plasma membrane excitability of the male gametes, its action must be linked to that of K⁺ channels. Several K⁺ channels exist in spermatozoa (Darszon et al. 1999, 2006), and future studies are necessary to determine if there is a specific functional relationship between α4 and one of the particular K⁺ channel expressed in sperm. An adequate membrane potential is essential for sperm motility, and cell membrane depolarization has been shown to be associated to infertility in asthenozoospermic patients (Calzada & Tellez 1997). Our results therefore suggest that one of the mechanisms by which the α4 isoform influences sperm motility is through its key role in maintaining the uneven transmembrane distribution of Na⁺ and K⁺, and the electrical potential of the sperm plasma membrane.

Besides its direct role in Na⁺ and K⁺ transport, we also found that the α4 isoform secondarily controls proton levels in spermatozoa, and that ouabain inhibition of α4 caused a pH decline of the sperm cytoplasm. This occurred after exposure of the cells to different physiological pH values, suggesting that α4 is able to prevent sperm acidification over a range of proton concentrations. The α4 isoform is not capable of directly transporting H⁺ (Blanco et al. 1999). Instead, and as has been shown for somatic cells (Bers et al. 2003, Despa et al. 2004), the Na,K-ATPase can functionally couple to the Na⁺/H⁺ exchanger (NHE) to regulate pH in sperm. In this manner, the α4 isoform provides spermatozoa with an inwardly directed Na⁺ gradient that provides the electrochemical energy to drive the secondary movement of protons out of the cells. This functional association between the α4 isoform and NHE is supported by several other lines of evidence. First, tissue-unspecific forms (NHE1 and NHE5) and sperm-specific types (sNHE and mtsNHE) of the NHE are expressed in spermatozoa (Darszon et al. 2001, Wang et al. 2003, 2007, Liu et al. 2010). Secondly, these exchangers are expressed in the mid- and principal segments of the sperm flagellum.
(Woo et al. 2002), thus co-localizing with the Na,K-ATPase α4 isoform (Woo et al. 2000, Wagoner et al. 2005, Sanchez et al. 2006), which will favor their cooperative action. Finally, the ionophores nigericin and monensin, which allow leakage of H⁺ out of the cells, are able to reestablish the motility that is lost in spermatozoa after ouabain inhibition of the Na,K-ATPase (Woo et al. 2002). Our results represent the first direct demonstration that activity of the Na,K-ATPase α4 isoform is linked to the control of H⁺ levels in spermatozoa. This mechanism will be important in preventing the rise of protons that takes place in the sperm cytoplasm as a consequence of active movement of the cells. Further studies will determine which of the several NHE systems are functionally coupled to the Na,K-ATPase α4 isoform. Changes in pH have been shown to influence sperm motility, and in detergent-extracted preparations of rat sperm, variations in the proton concentration have been shown to modulate sperm flagellar bending pattern and its response to cAMP and Ca²⁺ (Lindemann et al. 1991). Therefore, regulation of the proton concentration in the cells may represent one of the mechanisms by which the Na,K-ATPase α4 isoform influences sperm motility.

Our results also show that the activity of the α4 isoform is functionally coupled to the regulation of sperm [Ca²⁺]. Our experiments were performed in the absence of extracellular Ca²⁺. Therefore, the increase in [Ca²⁺], secondary to ouabain inhibition of α4 is not due to Ca²⁺ internalization from the media through plasma membrane uptake mechanisms, but is rather due to a decrease in the clearance of the cation from the cell cytoplasm. The only transport mechanism that has been described for the elimination of Ca²⁺ from the cytosol and that is linked to the function of the Na,K-ATPase is the Na⁺/Ca²⁺ exchanger (NCX; Lynch et al. 2008). In excitable cells, the function of the NCX heavily relies on the inward flux of Na⁺ maintained by the Na,K-ATPase to release Ca²⁺ out of the cytoplasm (Zhang et al. 2005). The effect we observed resembles observations made in excitable cells, and supports a functional coupling between the Na,K-ATPase and the NCX to maintain sperm intracellular Ca²⁺ low (Bers et al. 2006). This mechanism is particularly important in the regulation of [Ca²⁺] in smooth muscle cells of vessels and in cardiac cells, and it is responsible for the cardiotonic effects of digitalis in the heart (Blaustein et al. 1998, 2004). In these cells, the Na⁺-dependent regulation of [Ca²⁺], is mediated via a specific isoform of the Na,K-ATPase, the α2 polypeptide (Golovina et al. 2003, Zhang et al. 2005). Both the α2 and NCX are in proximity in a common subcellular domain, which favors the functional interaction between these ion transporters (Blaustein et al. 1998). The possibility of a mechanism for Ca²⁺ regulation based on the coupled activity of the α4 isoform and NCX is supported by the presence of a NCX transport system in spermatozoa (Wang et al. 2003). Interestingly, NCX has been shown to be expressed at the middle piece of the sperm flagellum (Krasznaï et al. 2006, Bedu-Addo et al. 2008), where the Na,K-ATPase α4 isoform is most abundant (Woo et al. 2000, Wagoner et al. 2005, Sanchez et al. 2006). Although additional experiments will be necessary to further define the combined function of the α4 isoform and NCX, our results suggest that, similar to the α2 isoform, the α4 polypeptide is another Na,K-ATPase isoform involved in Ca²⁺ regulation, in this case, specific to spermatozoa.

Figure 6 The Na,K-ATPase α4 isoform functions as a regulator of rat sperm [Ca²⁺]. Rat spermatozoa from the cauda epididymides were isolated and incubated for 30 min in modified Tyrode’s medium in the absence and presence of 10⁻⁶ and 10⁻³ M ouabain. Calcium green-AM was used to determine [Ca²⁺]. (A) Images from a representative cell from untreated controls or samples incubated with ouabain. Pictures were taken using a Nikon inverted confocal microscope, and analyzed using Metamorph software. Scale bar at the bottom right corresponds to 10 μm. (B) Compiled data from three different experiments. Bars represent the mean±S.E.M. Values significantly different from the untreated control are indicated with an asterisk, with P<0.001 for both 10⁻⁶ and 10⁻³ M ouabain versus untreated control. No statistical differences were found between samples containing 10⁻⁶ and 10⁻³ M ouabain (P=0.1).
Thus, α4 can be added to the list of ion transporters that control [Ca$^{2+}$]$_i$ in sperm (Bedu-Addo et al. 2008). Maintenance of sperm [Ca$^{2+}$], within a relative limited range is critical for the motility of the male gametes (Bedu-Addo et al. 2008). Studies performed in demembranated models show that changes in free calcium influence the curvature and symmetry of the sperm flagellum (Lindemann et al. 1992). In this manner, the ability of the α4 isoform to control [Ca$^{2+}$], may represent another mechanism by which this Na,K-ATPase polypeptide sustains sperm motility.

Previous work has studied the effects of ouabain in sperm of larger mammals. Specifically, ouabain has been reported to elicit a series of responses in bull spermatozoa. In this species, ouabain does not modify total motility or amplitude of lateral head displacement, but it decreases progressive motility, and curvilinear and average path velocities (Thundathil et al. 2006). On the other hand, McGrady (1979) found ouabain to cause a general decrease in bull sperm flagellar wave. Our results obtained from rats show that the inhibitory effect of ouabain extends to a wide range of sperm motility parameters. These disparities in results may depend on differences in the species studied, on the source of sperm (ejaculated versus epididymal), or on the experimental conditions used.

Besides its effects on sperm motility, ouabain has also been shown to produce capacitation in bull sperm through mechanisms that are independent of increases in intracellular Ca$^{2+}$ (Thundathil et al. 2006), but that involve activation of kinases and protein phosphorylation events in the cells (Newton et al. 2009). Our experiments were designed to investigate the rapid effects of ouabain and did not include long-term consequences on sperm capacitation. In contrast with the results in bulls, we found that ouabain inhibition of Na,K-ATPase in rat sperm induces a rise in intracellular Ca$^{2+}$. Increases in Ca$^{2+}$ have been reported as one of the biochemical changes that accompany rodent sperm capacitation (Visconti et al. 1998). Therefore, it is conceivable that the α4 isoform may play a potential role in the regulation of sperm capacitation in response to ouabain also in rats. In summary, it is clear that while some of the responses to ouabain are shared between bulls and rats, others are not. Further studies are required to ascertain these species-specific differences in the response of sperm to ouabain.

Our data indicate that the role of the Na,K-ATPase in sperm motility, membrane potential, intracellular pH, and [Ca$^{2+}$]$_i$ is isoform specific, and predominantly depends on the activity of the α4 rather than on that of the α1 polypeptide. We have shown previously that α4 exhibits unique enzymatic properties and a particular response to the transported ions (Blanco et al. 1999). In particular, when compared with the α1 isoform, α4 has a higher apparent affinity for intracellular Na$^+$, and small amounts of this cation selectively stimulate α4 function. Therefore, the α4 isoform appears to be better suited to maintain lower [Na$^+$], than α1. This predominant role of α4 in maintaining a steep Na$^+$ gradient across the sperm plasma membrane places the isoform as the primary Na,K-ATPase involved in sperm processes that depend on the Na$^+$ gradient. Our results also suggest that the α1 isoform may be playing a redundant role. Previous work showed that α1 contributes to approximately one-third of the total Na,K-ATPase activity of rat spermatozoa. In addition, α1 presents a broader distribution along the sperm flagellum compared with α4 (Wagoner et al. 2005). Therefore, although activity of α1 is not significantly involved in the sperm parameters studied in the present work, we cannot discard that it plays a role in the male gametes. In somatic cells, the widely expressed α1 isoform appears to perform a ‘housekeeping’ function, regulating the basal Na$^+$ and K$^+$ homeostasis in the cells, while the other isoforms carry out cell-specific tasks. In this manner, it is also possible that in spermatozoa, the α1 isoform has some ‘housekeeping’ role in maintaining the basal [Na$^+$]$_i$ gradient, while α4 is working to further control [Na$^+$]$_i$, and secondarily regulate other ion levels to sustain the high demands imposed by sperm motility.

Based on our observations, we propose the following model for the mechanism of action of the α4 isoform in the control of sperm motility. The ion transport activity of α4 is necessary to control the Na$^+$ and K$^+$ gradients that are important for the maintenance of the sperm plasma membrane potential in concert with K$^+$ channels. In addition, α4 generates the inward Na$^+$ flux that is secondarily used by the NHE and NCX transport systems to drive the movement of H$^+$ and Ca$^{2+}$ out of the cells respectively. The regulation of Na$^+$ along with the control of H$^+$ and Ca$^{2+}$ is crucial for sperm motility; therefore, the activity of α4 is of high relevance to the physiology of the male gametes.

In conclusion, our work provides new evidence for the mechanisms by which the Na,K-ATPase α4 isoform influences sperm motility. Future studies are underway to further elucidate the molecular basis of α4 role in the physiology and fertility of the male gametes.

Materials and Methods

Sperm preparation

All experimental protocols used in this work were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. Spermatozoa were obtained from the cauda of adult rat epididymis. For this, the epididymis was dissected, and its caudal portion was isolated and was cut at various points with a razor blade as described (Hernandez-Gonzalez et al. 2006). The tissue was placed in modified Tyrode’s medium (Ecroyd et al. 2004) containing 95 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 5.5 mM glucose, 0.27 mM pyruvic acid, 0.25 mM lactic acid, 40 mM HEPES, and 20 mM Tris (pH 7.4), and was incubated for 10 min at 37°C. After this time, spermatozoa were collected from the


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supernatant, centrifuged at 300 g for 30 s, and resuspended in modified Tyrode's medium. Cells were counted with the help of a hemocytometer, and were used for the different assays. All the experiments were conducted in modified Tyrode's medium.

**Sperm motility assays**

Approximately, 3 × 10⁶ cells were resuspended in 300 µl of the modified Tyrode's medium described above. Because extracellular Ca²⁺ is fundamental for sperm motility, 1.7 mM CaCl₂ was included in the medium. Cells in this solution were incubated without and with 10⁻⁶ or 10⁻³ M ouabain to selectively inhibit z4, or both the z₁ and z₄ isoforms (Wagoner et al. 2005). Incubation was done at 37 °C for different times and for a total of 2 h. Then, cells were labeled with 2 µl of a 75-µM stock of SITO 21, a green fluorescent nucleic acid stain, which helps tracking cell movement. After 2 min of incubation with the dye, 7-µl aliquots from each sample were taken and placed into glass cell chambers that were 20 µm in depth (Leja Products BV, Nieuw-Venmee, The Netherlands). The chambers were viewed using 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 CASA using the Minitube SpermVision Digital Semen Evaluation system (version 3.5; Penetrating Innovations, Verona, WI, USA). Different sperm motility parameters were analyzed, including total motility, progressive motility, curvilinear, average path, and straight line velocities, amplitude of lateral head displacement, beat cross frequency, and linearity. The analytical setup parameters used considered a cell identification or a cell size area between 15 and 900 µm², a cutoff velocity corresponding to a minimum straight line velocity of 5 µm/s, a progressive motility threshold corresponding to a straight line velocity of more than 20 µm/s, and a cutoff for low and medium average path velocities of 10 and 120 µm/s respectively. 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. An average of 50 cells/field was captured at a rate of 30 frames per field, and a total of ten fields in each sample were analyzed. Therefore, ~500 cells were analyzed per condition in each experiment. Each field was taken randomly by scanning the slide following a pre-established path to ensure consistency in the method. Experiments were repeated ten times; thus, allowing the observation of statistically significant numbers of cells in the assays. The obtained data were expressed as the percent of the motility of the untreated control at the initial time (5 min).

**Intracellular Na⁺ measurements**

Sperm intracellular Na⁺ determination was done using a fluorimetric method as described before (Hernandez-Gonzalez et al. 2006). Briefly, spermatozoa (20 × 10⁶ cells/ml) were incubated in modified Tyrode's medium for 15 min at 37 °C in the absence or presence of 10⁻⁶ M ouabain that inhibits only the z₄ isoform, and 10⁻³ M ouabain that blocks both the z₁ and z₄ isoforms (Wagoner et al. 2005). The cell-permeant non-fluorescent precursor of sodium green tetraacetate at a final concentration of 2.5 µM was added, and the samples were further incubated for another 30 min at room temperature. Cells were then washed twice by centrifugation at 300 g for 5 min and resuspended in 0.4 ml of fresh modified Tyrode's medium. Aliquots (50 µl each) were placed in cuvettes with a total volume of 2.5 ml of modified Tyrode's medium at room temperature, and fluorescence of each sample was measured under continuous stirring at an excitation/emission wavelength of 507/532 nm. Changes in fluorescence were expressed as the percent of the non-treated controls.

**Membrane potential assays in sperm**

Membrane potential was determined using the fluorescent indicator [DiSC₃(5)], which has been successfully used to measure membrane potential in mammalian spermatozoa (Plasek & Hrouda 1991, Espinosa & Darszon 1995, Zeng et al. 1995). [DiSC₃(5)] is charged and distributes across cellular membranes in response to electrochemical gradients. As a result of binding to intracellular proteins, the dye exhibits a slight shift in spectrum, providing reproducible estimates of plasma membrane potential. Because [DiSC₃(5)] also binds to mitochondria in their energized state, and this can interfere with changes in the fluorescence signal, the mitochondrial membrane potential was dissipated using carbonyl cyanide m-chlorophenylhydrazone (CCCP) as described (Demarco et al. 2003). Following this method, sperm samples containing 2 × 10⁶ cells/ml were treated with modified Tyrode’s medium for 20 min at 37 °C with and without ouabain in concentrations that inhibit only z4 (10⁻⁶ M), or both the z₁ and z₄ isoforms (10⁻³ M) (Wagoner et al. 2005). Then, [DiSC₃(5)], at a final concentration of 1 µM, was added, and the samples were incubated at 37 °C for an additional 8 min. Sperm were further incubated for another 2 min with CCCP at a final concentration of 1 µM (Hernandez-Gonzalez et al. 2006, 2007). After this time period, 2.5 ml of the suspension were transferred into a cuvette arranged with gentle stirring, and maintained at 37 °C. Then, fluorescence at an excitation/emission wavelength of 620/670 nm was recorded. After determining fluorescence of the different experimental conditions, calibration of the fluorescence changes into millivolts was done in the same sample by adjusting the membrane potential of the cells as described previously (Espinosa & Darszon 1995). Briefly, the K⁺ ionophore, valinomycin, was added at a final concentration of 1 µM to allow K⁺ to equilibrate across the plasma membrane. Then, the K⁺ concentration was increased stepwise to obtain final concentrations in the media of 9.9, 13.9, 21.9, and 37.9 mm KCl. After distribution of K⁺ in the cells, membrane potential follows the Nernst equilibrium and can be calculated. 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 previously (Plasek & Hrouda 1991, Espinosa & Darszon 1995, Zeng et al. 1995, Hernandez-Gonzalez et al. 2006).

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Intracellular pH measurements in sperm

Sperm intracellular pH was measured with the pH-sensitive dye SNARF-1-AM as described (Chow & Hedley 2007). Spermatozoa (20 × 10⁶ cells/ml) were incubated in modified Tyrode's medium adjusted at different pHs (7.0, 7.2, 7.4, and 7.6) for 15 min at 37 °C in the absence or presence of 10⁻⁶ and 10⁻³ M ouabain. Then, the cell-permeable non-fluorescent precursor SNARF-1-AM at a concentration of 5 μM was added, and the cells were incubated for another 30 min at 37 °C. After centrifugation at 300 g for 5 min, the cells were resuspended in 100 μl of fresh modified Tyrode's medium, and 15-μl aliquots were transferred to cuvettes with a total volume of 2.5 ml of modified Tyrode's medium. For the calibrating controls, parallel samples were placed in cuvettes with 2.5 ml of the above medium at preset pHs (6.0, 7.0, 7.2, 7.4, 7.6, and 8.0). These samples were further treated with 2 μg/ml of the ionophore nigericin for 20 min to permeabilize the cells and clamp the intracellular pH to the preset values in the medium (Chow & Hedley 2007). After this incubation time, sequential fluorescence of the experimental samples (untreated and ouabain-treated cells), followed by samples for pH calibration, was measured at an excitation of 488 nm and at an emission ratio of 640/580 nm. The pH-dependent spectral shift of SNARF-1 allowed determination of the cell pH based on the calibration curves obtained in medium with preset pHs as described (Chow & Hedley 2007).

Intracellular Ca²⁺ determination

Changes in [Ca²⁺], were determined using the cell-permeable fluorescent dye calcium green-1-AM (June et al. 2007). Cells were treated with modified Tyrode’s medium without Ca²⁺, and in the absence and presence of 10⁻⁶ or 10⁻³ M ouabain to inhibit either the α4 or the α1 isoform (Wagoner et al. 2005). During this incubation time, spermatozoa were loaded with Calcium Green-1-AM, which was used at a final concentration of 5 μM. After washing the cells twice in modified Tyrode’s medium by centrifuging at 300 g for 5 min, the cells were resuspended in fresh medium and placed on slides. To favor the adhesion to the slides, the cells were immobilized on Cell-Tak coated slides (BD Biosciences, Bedford, MA, USA) as described previously (O’Toole et al. 2000). Cells were then subjected to confocal microscopy. The fluorescence images of individual cells were collected using a Nikon scope and a 20× objective at an excitation/emission of 488/515–530 nm. The samples were maintained at 37 °C employing a heated device regulated by the system acquisition control. After the images were obtained, analysis of the collected data was done using Metamorph software (Molecular Devices, Downingtown, PA, USA). A total of 60 cells per experimental condition were analyzed in each experiment, and the results were expressed relative to the control, in which ouabain was omitted.

Statistical analysis

Experiments were repeated at least three times using a minimum of triplicate determinations. Statistical significance of differences between controls and ouabain-treated samples was determined by Student’s t-test using Sigma Plot software (Jandel Scientific, San Rafael, CA, USA). Statistical significance was defined as P<0.05.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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