ATPases, ion exchangers and human sperm motility

Rubén D Peralta-Arias[†], Carmen Y Vívienes, María I Camejo¹, Sandy Piñero¹, Teresa Proverbio, Elizabeth Martínez^{2,‡}, Reinaldo Marín and Fulgencio Proverbio

Laboratorio de Bioenergética Celular, Instituto Venezolano de Investigaciones Científicas (IVIC-CBB), AP 21827, Caracas 1020A, Venezuela, ¹Departamento de Biología de Organismos, Universidad Simón Bolívar, Caracas, Venezuela and ²Labofercla, Maternidad Leopoldo Aguerrevere, Caracas, Venezuela

Correspondence should be addressed to F Proverbio; Email: fproverb@yahoo.com; fproverb@ivic.gob.ve

[†]R D Peralta-Arias is now at Instituto Venezolano de Fertilidad, Valencia, Venezuela

[‡]E Martínez is now at Profertil, Clínica de Fertilidad, Barcelona, Venezuela

Abstract

Human sperm has several mechanisms to control its ionic milieu, such as the Na,K-ATPase (NKA), the Ca-ATPase of the plasma membrane (PMCA), the Na⁺/Ca²⁺-exchanger (NCX) and the Na⁺/H⁺-exchanger (NHE). On the other hand, the dynein-ATPase is the intracellular motor for sperm motility. In this work, we evaluated NKA, PMCA, NHE, NCX and dynein-ATPase activities in human sperm and investigated their correlation with sperm motility. Sperm motility was measured by Computer Assisted Semen Analysis. It was found that the NKA activity is inhibited by ouabain with two *K*_i (7.9×10^{-9} and 9.8×10^{-5} M), which is consistent with the presence of two isoforms of α subunit of the NKA in the sperm plasma membranes ($\alpha 1$ and $\alpha 4$), being $\alpha 4$ more sensitive to ouabain. The decrease in NKA activity is associated with a reduction in sperm motility. In addition, sperm motility was evaluated in the presence of known inhibitors of NHE, PMCA and NCX, such as amiloride, eosin, and KB-R7943, respectively, as well as in the presence of nigericin after incubation with ouabain. Amiloride, eosin and KB-R7943 significantly reduced sperm motility. Nigericin reversed the effect of ouabain and amiloride on sperm motility. Dynein-ATPase activity was inhibited by acidic pH and micromolar concentrations of Ca²⁺. We explain our results in terms of inhibition of the dynein-ATPase in the presence of higher cytosolic H⁺ and Ca²⁺, and therefore inhibition of sperm motility.

Reproduction (2015) **149** 475–484

Introduction

The human spermatozoon is a complex, minute and highly specialised cell responsible for the delivery of paternal DNA to the oocyte. Extensive work has been carried out to clarify the numerous physiological processes that occur in the cell that allows it to achieve fertilisation (capacitation, hyperactivation, acrosome reaction, fusion, etc.). In this context, different ATPases play a crucial role in sperm physiology.

After leaving the testes, spermatozoa are morphologically differentiated but immotile and unable to fertilise. The initiation and maintaining of motility is acquired during transit through the epididymis (principal reservoir of sperm), being enhanced after ejaculation (Marín-Briggiler *et al.* 2005). Motility could be modulated by many factors such as intracellular pH (Giroux-Widemann *et al.* 1991, García & Meizel 1999), the Na,K-ATPase (NKA; Koçak-Toker *et al.* 2002, Sanchez *et al.* 2006), the plasma membrane Ca²⁺ pump (Williams & Ford 2003, Okunade *et al.* 2004, Schuh *et al.* 2004), the Na⁺/H⁺-exchanger (NHE; Wong *et al.* 1981, Woo *et al.* 2002), the Na⁺/Ca²⁺-exchanger

(NCX; Krasznai *et al.* 2006) and the dynein-ATPase (Lindemann 2003). NKA activity has been found in human spermatozoa, and its inhibition produces a significant reduction in sperm motility (Koçak-Toker *et al.* 2002). This enzyme catalyses the exchange of cytoplasmic Na⁺ for extracellular K⁺ in a 3:2 ratio (Kaplan 2002). The α subunit of the NKA is responsible for the catalytic and transport properties of the enzyme and contains the binding sites for cations, ATP and ouabain (Lingrel & Kuntzweiler 1994). Until now, only the $\alpha 1$ and $\alpha 4$ isoforms have been identified in the midpiece of the sperm, being $\alpha 4$ the more physiologically relevant because it represents a significant portion of the total NKA relevant population in human and mouse sperm (Blanco *et al.* 2000, Woo *et al.* 2002, Hlivko *et al.* 2006, Sanchez *et al.* 2006). A distinctive feature of $\alpha 4$ is that it is much more sensitive to ouabain than the $\alpha 1$ isoform (0.5×10^{-9} and 4.6×10^{-7} M ouabain respectively; Sanchez *et al.* 2006). Mouse and human sperm incubated with ouabain have shown that selective inhibition of $\alpha 4$ is sufficient to significantly decrease sperm motility (Koçak-Toker *et al.* 2002, Woo *et al.* 2002, Sanchez *et al.* 2006).

NKA generates a Na^+ gradient across the plasma membrane, providing the energy required for the normal function of the NHE (Wong *et al.* 1981). NHE exchanges H^+ from the cell for extracellular Na^+ , and plays an important role in the regulation of intracellular pH (Hamamah & Gatti 1998). It is well known that acidic intracellular pH leads to a reduction in sperm motility (Wong *et al.* 1981, Giroux-Widemann *et al.* 1991). Amiloride, an inhibitor of NHE, is able to decrease sperm motility in rats (Wong *et al.* 1978).

The plasma membrane Ca^{2+} pump (PMCA) is an ATPase that extrudes Ca^{2+} from the cell (Salvador *et al.* 1998), and is involved in the fine control of the intracellular Ca^{2+} concentration. ATP2B4 is the major isoform in sperm, which is found primarily in the sperm flagellum and is required for sperm motility (Wennemuth *et al.* 2003, Okunade *et al.* 2004, Aravindan *et al.* 2012). Previous data show that ablation of the *ATP2B4* gene leads to a decrease in sperm motility and infertility (Okunade *et al.* 2004, Schuh *et al.* 2004). Human sperm incubated with quercetin (an inhibitor of PMCA) show a reduction in motility and this correlates with an increase in the intracellular concentrations of Ca^{2+} from a resting value of 1.4×10^{-7} M up to 1.28×10^{-6} M (Williams & Ford 2003).

The NCX is expressed in the plasma membrane of human sperm, specifically in the acrosome and the midpiece (Krasznai *et al.* 2006). Inhibition of the NCX with different drugs (bepridil, DCB and KB-R7943) leads to a rise in intracellular Ca^{2+} and significant inhibition of human sperm motility (Krasznai *et al.* 2006).

Dyneins are proteins working as intracellular motors that project from the peripheral doublets of the axoneme arms. They both play a crucial role in tubular sliding for the generation of sperm motility. The absence of the dynein arms, seen in some syndromes, leads to impairment of sperm motility and male infertility (Afzelius *et al.* 1975, Jouannet *et al.* 1983, Goodenough & Heuser 1985, Neesen *et al.* 2001). Moreover, Gibbons (1965, 1974) demonstrated that dyneins exhibit ATPase activity, and our group has recently developed a straightforward assay to test dynein-ATPase activity (Vivenes *et al.* 2009). This dynein-ATPase is Mg^{2+} -dependent and is inhibited by micromolar amounts of vanadate (Gibbons & Gibbons 1987, Shpetner *et al.* 1988).

In this work, we studied the effect of different inhibitors of NKA, PMCA, NHE and NCX and their influence on the motility of human sperm. We have also analysed the effect of different concentrations of free Ca^{2+} on the dynein-ATPase activity of human sperm. Together, these data contribute to understand the relationship between pH, Ca^{2+} and dyneins, giving a novel approach to how ATPases and ion exchangers work closely to maintain sperm motility.

Materials and methods

Semen samples

Semen samples were obtained by masturbation into a sterile plastic container from healthy donors with normal semen parameters in agreement with the guidelines of the World Health Organisation (2010) and in accordance with the ethical standards established by the Declaration of Helsinki, as revised in Tokyo 2004. The subjects were requested to have 3–4 days of abstinence before the collection. The median abstinence time was 3 days. The semen samples were analysed with respect to semen volume, sperm motility and sperm concentration. Analysis was started as soon as the ejaculates had liquefied. All ejaculates liquefied within 45–60 min. The semen samples were used either to test the sperm motility under the different incubation conditions or to isolate the sperm plasma membrane and axoneme fractions. The study protocol was approved by the Bioethics Committee of IVIC, and all donors gave informed signed consent.

Sperm motility

Motile spermatozoa were selected from 1 ml of liquefied semen by centrifugation (600 *g* for 10 min) through a discontinuous Sydney IVF Sperm Gradient (Cook Medical, Bloomington, IN, USA) (0.5 ml of 80% and 0.5 ml of 40%). The supernatant was discarded and the pellet was resuspended with 1 ml of Ham's F-10 (Sigma Chemical), at pH 7.8. After another centrifugation (600 *g* for 5 min), the supernatant was discarded and the pellet was resuspended in 0.5 ml Ham's F-10. The sperm concentration ranged from 3 to 6 millions/ml. The sperm concentration and motility were determined by Computer Assisted Semen Analysis (CASA; Hamilton Thorne, Beverly, MA, USA). The experiments were carried out by incubating 100 μl of motile spermatozoa with 100 μl of Ham's F-10 at 37 °C, and in agreement with the experimental protocol, in the presence and absence of ouabain, amiloride, nigericin, eosin and KB-R7943 (Sigma Chemical). The sperm motility (as percentage of forward progressives from the total sperm cells analysed), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), beat cross frequency (BCF) and amplitude of lateral head displacement (ALH) were determined.

Isolation of sperm plasma membranes and axoneme-containing fractions

Sperm plasma membranes and axoneme-containing fractions were obtained by a modification of a method previously described by Olson *et al.* (1987) and Vivenes *et al.* (2009). After liquefaction, the semen samples were resuspended with 9 ml of buffer A (50 mM Tris-HCl, pH 7.4 at 4 °C; 100 mM NaCl). The suspension was then centrifuged at 59 800 *g*, for 10 min at 4 °C with a SW41Ti rotor in a Beckman Ultracentrifuge. The supernatant was discarded and the pellet was resuspended in buffer B (10 mM Tris-HCl, pH 7.4 at 4 °C; 0.5 mM EDTA with a protease inhibitor cocktail (0.5 mM PMSF, 5 mM benzamidin, 1 $\mu\text{g/ml}$ leupeptin and 1 $\mu\text{g/ml}$ pepstatin A)). The suspension was homogenised using a high-performance disperser

Ultraturrax T-25 (IKA) with a S25N-18G dispersing element at 13 500 r.p.m. for 5 s twice. Then, 3 ml of the suspension was placed on top of a discontinuous gradient of sucrose (3 ml 50% and 3 ml 20%). The gradient was centrifuged at 111 132 *g* for 60 min with a SW41Ti rotor in a Beckman Ultracentrifuge. The pellet was washed and resuspended in buffer B and assayed for dynein-ATPase activity. The band at the 20–50% interface was removed, washed, resuspended with buffer B and recentrifuged (111 132 *g*, SW41Ti, for 90 min). The supernatant was discarded and the pellet (containing the plasma membrane enriched fraction) was resuspended in buffer B and assayed for NKA and PMCA activities.

SDS pre-treatment of the sperm fraction

In order to avoid the presence of membrane vesicles, the sperm fractions were pretreated with SDS/BSA/Imidazole as previously described (Marín *et al.* 1986). A 240 µl aliquot of the fraction (~0.4 mg protein/ml) was pretreated in a solution giving a final concentration of 0.05% SDS, 0.025% BSA, 0.625 mM imidazole, pH 7.2 at 37 °C. The optimal SDS/protein ratio was around 0.625 µg SDS/µg protein. The fractions were incubated for 20 min at 37 °C, and then assayed for ATPase activity. Protein was determined by the Bio-Rad micromethod (Bio-Rad Laboratories) based on Bradford's assay (Bradford 1976).

ATPase assays

The ATPase activity was determined by following an experimental protocol similar to the one described elsewhere (Marín *et al.* 1986, Proverbio *et al.* 1986). A 180 µl aliquot of the specific incubation medium was prewarmed at 37 °C for 2 min. The reaction was started by addition of 20 µl of the sperm fraction (0.1–0.2 mg protein ml/l) previously treated with SDS. After a 30 min incubation, 300 µl of a stop solution containing (final concentrations) 0.72% SDS, 0.48% molybdic acid, 2.8% ascorbic acid and 2.18% HCl acid were added to the test tubes, which were placed on ice for 10 min, and then mixed with 500 µl of a solution containing (final concentrations) 2% sodium arsenite, 2% sodium citrate and 2% acetic acid. The tubes were then rewarmed at 37 °C for 10 min, and the developed colour was measured in a Sunrise (Tecan Systems Inc., San Jose, CA, USA) spectrophotometer at 705 nm. All samples were run in quadruplicate. Activity was expressed as nmoles of P_i liberated/mg of protein per min, after subtraction of a blank run in parallel without the membrane suspension, which was added after the reaction was stopped. ATPase activity was time-linear for all the samples assayed using this 30 min incubation time.

NKA activity was assayed with the following incubation medium (final concentrations): 50 mM Tris-HCl, pH 7.0 at 37 °C; 2 mM $MgCl_2$; 100 mM NaCl; 20 mM KCl and 2 mM Tris-ATP, with and without 2×10^{-3} M ouabain. Na,K-ATPase activity was calculated as the difference between the amount of phosphate liberated in the test tubes in the presence and absence of ouabain.

The dynein-ATPase activity was assayed with the following incubation medium (final concentrations): 50 mM Tris-HCl,

pH 8.0 at 37 °C; 2 mM $MgCl_2$; 2 mM Tris-ATP; 2×10^{-3} M ouabain, 1 µM oligomycin, 1 µM thapsigargin, with and without 5 µM vanadate. The dynein-ATPase activity was calculated as the difference between the amount of phosphate liberated in the presence and absence of 5 µM vanadate (as sodium orthovanadate). The inhibitory effect of vanadate on the ATPase activity was completely reversed by the presence of 1 mM norepinephrine in the incubation medium. The dynein-ATPase activity was inhibited by 6 mM LiCl (Vivenes *et al.* 2009).

Statistical analysis

The results are expressed as mean $\pm$ S.E.M. for the number of indicated assays in each case. The S.E.M. of the differences were calculated for paired data. Data analysis was performed with the Student's *t*-test, being '*n*' the number of repeated assays with different preparations. The multiple comparisons for the same incubation time were performed with Dunnett's test. Only probabilities <0.05 were accepted.

Results

NKA and human sperm motility

A distinctive feature of NKA activity in human and rodent sperm is the presence of the $\alpha 1$ and $\alpha 4$ isoforms. The $\alpha 4$ isoform represents 80 and 44% of the total NKA population found in mouse and human sperm respectively (Blanco *et al.* 2000, Sanchez *et al.* 2006). Remarkably, the $\alpha 4$ isoform is much more sensitive to ouabain than $\alpha 1$, both in humans and rodents (Woo *et al.* 2002, Sanchez *et al.* 2006). Therefore, we tested the effect of three different concentrations of ouabain (1×10^{-6} , 1×10^{-4} and 2×10^{-3} M), in order to determine the role of the NKA subunits in sperm motility. In Fig. 1, we can see that all the tested concentrations of ouabain inhibit sperm motility in a similar fashion. Because the $\alpha 4$ isoform of the NKA is much more sensitive to ouabain than the $\alpha 1$ isoform ($K_i = 0.5 \times 10^{-9}$ and 4.6×10^{-7} M ouabain respectively; Sanchez *et al.* 2006), the lowest concentration of ouabain used in the experiments shown in Fig. 1 (1×10^{-4} M) seems to be sufficient to partially inhibit the $\alpha 1$ isoform. Therefore, we tested the effect of 1×10^{-6} M ouabain on sperm motility. It was found that a 180 min incubation of the motile spermatozoa with 1×10^{-6} M ouabain produced a significant reduction in sperm motility ($79 \pm 3\%$ forward progressives, control vs $48 \pm 4\%$ forward progressives, 1×10^{-6} M ouabain; $n=3$, $P<0.01$). In other words, the inhibition of the $\alpha 4$ isoform alone is enough to partially inhibit the motility of human sperm.

The presence of the NKA was evaluated in a plasma membrane-enriched fraction of human sperm. As given in Table 1, there is a significant Mg^{2+} -dependent and ouabain-sensitive NKA activity. In order to be sure that the inhibition of this ATPase activity is complete, we used an excess of ouabain (2×10^{-3} M). We show that

Figure 1 Functional inhibition of sperm motility by ouabain. The percentage of forward progressives with no ouabain (control), 1×10^{-6} M ouabain, 1×10^{-4} M ouabain or 2×10^{-3} M ouabain was determined at different incubation times up to 180 min. The data presented in each curve are the results of human sperm from eight different donors, expressed as means $\pm$ S.E.M. The multiple comparisons for the same incubation time were performed with Dunnet's test. $^a P < 0.001$ vs control.

the inhibition is complete, because the Na+K-stimulated ATPase activity is similar to that of the ouabain-sensitive ATPase activity (Table 1). The sensitivity to ouabain of this NKA activity was further evaluated. Figure 2 shows the effect of different concentrations of ouabain on NKA activity in plasma membrane-enriched fractions of human sperm. It can be seen that inhibition of NKA activity is clearly biphasic, corresponding to two components with different sensitivity to the drug. Each component was analysed separately, giving two K_i for ouabain, 7.9×10^{-9} and 9.8×10^{-5} M. These values are in agreement with previously reported concentrations required for selective inhibition of the $\alpha 1$ and $\alpha 4$ isoforms of NKA in human sperm (Sanchez *et al.* 2006).

NHE and human sperm motility

It has been proposed that the inhibitory effect of ouabain on sperm motility is due to the reduction in the Na^+ gradient across the plasma membrane, being critical for the normal functioning of transporters such as NHE (Garcia & Meizel 1999). This would alter the intracellular pH and, consequently dynein-ATPase activity, which has been shown to be inhibited when the intracellular pH is acidified, affecting in this way sperm motility (Vivenes *et al.* 2009). Consequently, if a proton ionophore, such as nigericin, is added after the incubation with 1×10^{-4} M ouabain, the intracellular pH could be alkalised and the sperm motility could also be restored (Woo *et al.* 2002). This experiment was

carried out and the results are shown in Fig. 3A. It is noticeable how the addition of 6.25×10^{-6} M nigericin to human sperm incubated with 1×10^{-4} M ouabain restores their motility levels almost immediately. The recovery of sperm motility did not change even after 30 min of incubation with nigericin (data not shown). In another set of experiments, we incubated human sperm with amiloride, a well-known inhibitor of the NHE that induces acidification of the intracellular milieu in human sperm cells (Garcia & Meizel 1999), and then restoring the intracellular pH with nigericin. The results of these experiments are shown in Fig. 3B. Addition of 5×10^{-4} M amiloride gave similar results to 1×10^{-4} M ouabain, inhibiting of sperm motility, which is partially restored by the addition of nigericin. The effect of nigericin did not change after 30 min of incubation (data not shown).

PMCA, NCX and human sperm motility

The importance of Ca^{2+} ions in the regulation of diverse processes in sperm, including motility and acrosome reaction, has been well documented (Jimenez-Gonzalez *et al.* 2006). It is known that elevated cytosolic Ca^{2+} inhibits sperm motility and yet low levels permit or promote motility. PMCA, NCX and SERCA are the main mechanisms that participate in the control of cytoplasmic Ca^{2+} concentrations (Lawson *et al.* 2007). In addition, loss of ATP2B4 causes a sperm motility defect and male infertility (Schuh *et al.* 2004). However, the exact mechanisms involved in the control of sperm motility by cytosolic Ca^{2+} remain largely unknown. In the current study, we used eosin, a known inhibitor of ATP2B4 (Gatto & Milanick 1993, Kennedy & Mangini 1996, Mohamed *et al.* 2013) and KB-R7943, a known inhibitor of NCX (Krasznai *et al.* 2006), in order to evaluate their effects on sperm motility. Figure 4 shows that sperm motility decreases in the presence of either 2.5×10^{-5} M eosin ($38 \pm 2\%$ at 180 min of incubation, $P < 0.001$) or 2.5×10^{-5} M KB-R7943 ($37 \pm 2\%$ at 180 min of incubation, $P < 0.001$).

In order to gauge whether the effects of ouabain, eosin and KB-R7943 on sperm motility were produced through the same mechanism, we studied the effect of these drugs

Table 1 NKA activity of plasma membrane enriched fractions of human sperm.

Incubation medium	ATPase activity (nmol Pi/mg prot. min)	NKA
(a) $\text{Mg}^{2+} + \text{Na}^+ + \text{ouabain}$	272 ± 8	
(b) $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$	371 ± 12	$99 \pm 6^*$ (b and a)
(c) $\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+ + \text{ouabain}$	274 ± 9	$97 \pm 7^*$ (b and c)

Assays made according to the 'Materials and methods' section. The ouabain concentration used was 2×10^{-3} M. Activity values expressed as mean $\pm$ S.E.M., $n = 12$. The S.E.M. for the NKA activity was calculated for paired data ($^* P < 0.001$).

Figure 2 Dose-response curve for ouabain inhibition of NKA activity of the plasma membrane-enriched fraction of human sperm. Specific activity was determined on sperm plasma membranes as described in 'Materials and methods' section in the absence and presence of the indicated concentrations of ouabain. Maximal NKA activity was determined from the difference between the Na^+ and K^+ -dependent hydrolysis of ATP in the absence and presence of 2×10^{-3} M ouabain. Results are expressed as percentage of maximal activity. The curve represents the best fit of the experimental data, using the double Boltzmann function. Each value represents the mean $\pm$ S.E.M. of quadruplicate determinations from 20 separate experiments with different preparations.

combined on the human sperm motility. Figure 5 shows that the combination of ouabain with either eosin or KB-R7943 produces a dramatic decrease in sperm motility (10 ± 1 and $6 \pm 1\%$ at 180 min for ouabain + eosin and ouabain + KB-R7943, respectively, $P < 0.001$). Under these conditions, motility declines over a shorter period of time, and at 180 min, the percentage of forward progressives almost reaches zero, confirming the additive action of the drugs.

NKA, PMCA, NHE, NCX and human sperm motility

Sperm motility, shown in Figs 1, 3, 4 and 5, was evaluated by measuring the percentage of forward progressives (a+b). However, there are other CASA parameters that have been related to sperm motility. In order to test whether the different CASA parameters related to sperm motility are affected by the different inhibitors of NKA, PMCA, NHE and NCX, we incubated motile sperms with these inhibitors for 180 min. At the end of the incubation period, we measured the different CASA parameters related to sperm motility and the results are given in Table 2. Notice that the inhibition of the NKA and NHE only affects the percentage of forward progressives, while the other CASA parameters remain practically unaffected. This is not the case when the PMCA and NCX are inhibited. Under these conditions, all the CASA parameters are significantly reduced.

Dynein-ATPase activity and calcium

Dynein-ATPase activity was assayed in an axoneme-containing fraction of human sperm and identified as: i) Mg^{2+} -dependent; ii) ouabain-insensitive; iii) oligomycin-insensitive and iv) inhibited by vanadate which was

Figure 3 (A) Partial reversal of the effect of ouabain by nigericin on human sperm motility. After 180 min of ouabain exposure (1×10^{-4} M), 6.25×10^{-6} M nigericin was added to the incubation medium and after three additional minutes of incubation motility was assessed. The data presented in each curve are the results of human sperm from eight different donors, expressed as means $\pm$ S.E.M. The multiple comparisons for the same incubation time were performed with Dunnet's test. $^a P < 0.001$ vs control. (B) Partial reversal of the effect of amiloride by nigericin on human sperm motility. After 120 min of amiloride exposure (5×10^{-4} M), 6.25×10^{-6} M nigericin was added to the incubation medium and after five additional minutes of incubation motility was assessed. The data presented in each curve are the results of human sperm from six different donors, expressed as means $\pm$ S.E.M. The multiple comparisons for the same incubation time were performed with Dunnet's test. $^a P < 0.001$ vs control and $^b P < 0.001$ vs 180 min amiloride.

Figure 4 (A) Functional inhibition of sperm motility by eosin. The percentage of forward progressives with no eosin (control) or 2.5×10^{-5} M eosin was determined at different incubation times up to 180 min. The data presented in each curve are the results of human sperm from eight different donors, expressed as means $\pm$ S.E.M. The multiple comparisons for the same incubation time were performed with Dunnet's test. ^a $P < 0.001$ vs control. (B) Functional inhibition of sperm motility by KB-R7943. The percentage of forward progressives with no KB-R7943 (control) or 2.5×10^{-5} M KB-R7943 was determined at different incubation times up to 180 min. The data presented in each curve are the results of human sperm from eight different donors, expressed as means $\pm$ S.E.M. The multiple comparisons for the same incubation time were performed with Dunnet's test. ^a $P < 0.001$ vs control.

reversed in the presence of norepinephrine (Vivenes *et al.* 2009). Figure 6 shows the effect of free Ca^{2+} concentrations on dynein-ATPase activity. While concentrations below 1×10^{-7} M free Ca^{2+} do not affect dynein-ATPase activity, concentrations above this value significantly decrease it.

Discussion

Despite the existence of studies demonstrating the influence of NKA, PMCA and NCX on human sperm

behaviour, the key target where these physiological mechanisms act controlling or changing sperm motility is unknown. Our experiments with ouabain and nigericin show that this ionophore partially reverses the ouabain-induced inhibition of sperm motility.

Figure 5 (A) Functional inhibition of sperm motility by ouabain, eosin or ouabain + eosin. The percentage of forward progressives with no inhibitors (control), 1×10^{-4} M ouabain and 2.5×10^{-5} M eosin or both inhibitors together (1×10^{-4} M ouabain and 2.5×10^{-5} M eosin) was determined at different incubation times up to 180 min. The data presented in each curve are the results of human sperm from eight different donors, expressed as means $\pm$ S.E.M. The multiple comparisons for the same incubation time were performed with Dunnet's test. ^a $P < 0.001$ vs control and ^b $P < 0.001$ vs control, vs 1×10^{-4} M ouabain and vs 2.5×10^{-5} M eosin. (B) Functional inhibition of sperm motility by ouabain, KB-R7943 or ouabain + KB-R7943. The percentage of forward progressives with no inhibitors (control), 1×10^{-4} M ouabain, 2.5×10^{-5} M KB-R7943 or both inhibitors together (1×10^{-4} M ouabain, 2.5×10^{-5} M KB-R7943) was determined at different incubation times up to 180 min. The data presented in each curve are the results of human sperm from eight different donors, expressed as means $\pm$ S.E.M. The multiple comparisons for the same incubation time were performed with Dunnet's test. ^a $P < 0.001$ vs control and ^b $P < 0.001$ vs control, vs 1×10^{-4} M ouabain and vs 2.5×10^{-5} M KB-R7943.

Table 2 Comparison of the effects of several inhibitors on the kinematic parameters in human semen.

CASA parameter	Incubation condition					
	Control	+ ouabain	+ eosin	+ ouabain + eosin	+ amiloride	+ KB-R7943
% FP	79 ± 3	48 ± 4*	38 ± 2*	11 ± 1*	46 ± 3*	47 ± 2*
VCL (µm/s)	96 ± 4	98 ± 3	40 ± 2*	37 ± 1*	97 ± 2	49 ± 4*
VSL (µm/s)	42 ± 3	39 ± 3	22 ± 1*	25 ± 1*	40 ± 2	30 ± 2 [†]
VAP (µm/s)	45 ± 2	42 ± 1	23 ± 2*	26 ± 1*	43 ± 2	21 ± 2*
BCF (beats/s)	15 ± 1	14 ± 2	7 ± 1*	6 ± 1*	14 ± 1	8 ± 2 [†]
ALH (µm)	4.0 ± 0.2	4.2 ± 0.1	2.6 ± 0.1*	2.5 ± 0.1*	4.3 ± 0.2	3.1 ± 0.1 [†]

Assays made according to the 'Materials and methods' section. The experiments were carried out by incubating 100 µl of motile spermatozoa with 100 µl of Ham's F-10 at 37 °C, and in agreement with the experimental protocol, in the presence and absence of 1×10^{-6} M ouabain, 2.5×10^{-5} M eosin, the combination of ouabain + eosin, 5×10^{-4} M amiloride and 2.5×10^{-5} M KB-R7943. The CASA parameters related to sperm motility were then determined after 180 min of incubation. The values are expressed as mean ± S.E.M., $n=8$. * $P<0.001$ vs control and [†] $P<0.01$ vs control. %FP, % forward progressives; VAP, averaged path velocity; VSL, straight line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

That supports the idea that the ultimate effect of ouabain is on the dissipation of the Na^+ gradient across the plasma membrane (generated by the NKA activity), leading to inhibition of NHE activity and therefore acidification of the intracellular milieu (Figs 1 and 3). This conclusion was supported by the results obtained with amiloride, a NHE inhibitor, where nigericin also reactivated the sperm motility (Fig. 3). Although we did not measure intracellular pH, our conclusions are in agreement with previous studies in human sperm that measured the intracellular pH of sperm after incubation with either ouabain or amiloride (Garcia & Meizel 1999, Carrasquel *et al.* 2009). Amiloride (5×10^{-4} M), under the same incubation conditions used in our study, significantly decreases intracellular pH in human sperm (Carrasquel *et al.* 2009). The $\alpha 1$ and $\alpha 4$ isoforms of NKA are expressed in the midpiece of human sperm (Hlivko *et al.* 2006), and selective inhibition of $\alpha 4$ alone causes drastic loss of motility (Hlivko *et al.* 2006, Sanchez *et al.* 2006). The biochemical evidence presented in this study confirms the existence of both isoforms in human sperm and also demonstrates their role in sperm motility.

Interestingly, nigericin did not completely restore sperm motility after incubation with either ouabain or amiloride, indicating that another factor could be participating in the regulation of motility. Taking into account the well-documented importance of Ca^{2+} ions in the regulation of diverse processes in sperm, including motility and acrosome reaction (Yanagimachi & Usui 1974, Young & Nelson 1974, Wennemuth *et al.* 2003), we decided to alter some mechanisms responsible for the intracellular concentrations of Ca^{2+} , and to evaluate their effect on sperm motility. Our data indicate that specific inhibition of either PMCA or NCX affects motility (Fig. 4), which is in agreement with previous studies (Williams & Ford 2003, Schuh *et al.* 2004, Krasznai *et al.* 2006, Carrasquel *et al.* 2009). Furthermore, the combination of both ouabain and either eosin (PMCA inhibitor) or KB-R7943 (NCX inhibitor) produces an additive effect on sperm motility (Fig. 5). This suggests

that the effects of intracellular pH and Ca^{2+} on human sperm motility are independent of each other. In this regard, the data in Table 2, indicating that the CASA parameters related to sperm motility are differentially affected by intracellular pH and Ca^{2+} , is in agreement with the conclusion that the decrease in pH produced by inhibition of NKA or NHE only diminishes the percentage of forward progressives, while the rise in intracellular $[\text{Ca}^{2+}]$ produced by inhibition of PMCA or NCX, affects all the measured CASA parameters.

Sperm motility is proportional to the number of force-generating dynein molecules present in the

Figure 6 Effect of different free Ca^{2+} concentrations on the dynein-ATPase activity of the axoneme-containing fraction of human sperm. The dynein-ATPase activity was as described by Vivenes *et al.* (2009). The axoneme-containing fraction of human sperm was assayed in an incubation medium containing 50 mM Tris-HCl, pH 8.0 at 37 °C; 2 mM MgCl_2 ; 2 mM Tris-ATP; 2 mM ouabain, 1 µM oligomycin, 1 µM thapsigargin, with and without 5 µM vanadate. The dynein-ATPase activity was calculated as the difference between the amount of phosphate liberated in the presence and absence of 5 µM vanadate (as sodium orthovanadate). The values expressed as mean ± S.E.M. for $n=17$. ^a $P<0.02$ vs 0 free $[\text{Ca}^{2+}]$ and ^b $P<0.01$ vs 0 free $[\text{Ca}^{2+}]$.

axoneme (Lindemann 2003). This is regulated by phosphorylation–dephosphorylation processes in the outer dynein arm (ODA), through AMP_c-dependent kinases and calmodulin-dependent phosphatases (Lindemann & Kanous 1989, Tash & Bracho 1994). Dyneins are the most important intracellular motors for generation of sperm motility, so we focused our study on trying to demonstrate the effect of pH and free Ca²⁺ on dynein-ATPase activity. We have previously shown that this activity has an optimal pH value around 8.0, and that acidic pH values are inhibitory (Vivenes *et al.* 2009). Interestingly, in the cross-talk between internal/external pH regulation and calcium-dependent sperm processes, it is important to mention that PMCA exchanges one Ca²⁺ for one H⁺ and consequently its inhibition could modify the pH of the cytosol (Di Leva *et al.* 2008) and therefore the sperm motility. In addition, in this study, we show that free Ca²⁺ concentrations above 1×10^{-7} M are also inhibitory (Fig. 6). In this regard, it is known that the inhibition of both PMCA and NCX results in an increase in the intracellular free Ca²⁺ from 1×10^{-7} M (resting concentration) to values between 2.5×10^{-7} and 5×10^{-7} M (Williams & Ford 2003, Krasznai *et al.* 2006, Carrasquel *et al.* 2009).

It is important to mention a study on human sperm from patients with a pathology known as lacking ODAs (LODA; Keskes *et al.* 1998). This condition is associated with a lower percentage of motile spermatozoa in semen, due to the absence of external dynein arms. These spermatozoa can be reactivated after being demembrated and incubated with appropriate concentrations of Mg²⁺-ATP. The reactivation profile of LODA spermatozoa as a function of Mg²⁺-ATP concentration was not the same as that of normal spermatozoa. In fact, no change in the reactivation level of LODA spermatozoa was found when the pH was increased from 7.1 to 7.8 unlike normal spermatozoa, whose reactivation level was significantly increased at pH 7.8 (Keskes *et al.* 1998). However, the motility of LODA spermatozoa could be partially reinitiated with appropriate concentrations of Mg²⁺-ATP and removal of the free Ca²⁺ with EGTA, concluding that reactivation of LODA spermatozoa is pH-independent but Ca-dependent (Keskes *et al.* 1998). These data and our results (Table 2) could support the idea that the dyneins present a 'pH sensor' located in the inner dynein arm (IDA), and a 'Ca²⁺ sensor' located in the ODA. However, we cannot disregard the possibility that IDA presents a certain degree of pH sensitivity, or that the inhibition of ODA activity at acidic pH could thus negatively affect the IDA. A putative cooperation between the two arms has been suggested, which would be operated by means of a dynein regulatory complex (Kamiya *et al.* 1989, Piperno *et al.* 1992).

Finally, we would like to comment on the role of pH, Na⁺ and Ca²⁺ within the epididymis. Spermatozoa, as

well as their surrounding fluid, initially elaborated by the seminiferous tubules, undergo numerous changes during their passage through the entire length of the epididymal structures where they are thought to be largely immotile (Yeung & Cooper 2002). Changes in pH, Na⁺ and Ca²⁺ can be summarised as follows:

- i) The extracellular Na⁺ concentration decreases progressively from the caput to the cauda, whereas the K⁺ concentration is increased in the same order, being the cauda where most of the water and Na⁺ absorption occurs (Wong *et al.* 1978). It has been estimated that 76% of epididymal Na⁺ is reabsorbed from the caput to the cauda (Wong *et al.* 1978), producing the following concentrations of Na⁺: (a) 135 mM in the seminiferous tubules; (b) 104 mM in the caput and (c) 37 mM in the distal cauda (Verma 2001). The dramatic fall in extracellular Na⁺ would induce the inhibition of both NHE and NCX, with subsequent accumulation of excess H⁺ and Ca²⁺ in the sperm cytoplasm, followed by inhibition of the dynein-ATPase by means of the sensors located on ODAs and IDAs, and therefore inhibition of motility.
- ii) Cytoplasmic Ca²⁺ in sperm from the caput epididymidis is two to six times higher than that in sperm from the cauda region (Vijayaraghavan & Hoskins 1990). It has been suggested that this could be due to sperm acquisition of ATP2B4 from membranous vesicles in the epididymal luminal fluid during sperm transit throughout the epididymis (Patel *et al.* 2013). The increase in PMCA molecules would lower the cytoplasmic Ca²⁺ and therefore it could promote motility initiation in cauda sperm. However, the PMCA activity in caudal sperm could be partially inhibited because of low intracellular pH which would contribute to keep the cytoplasmic Ca²⁺ in an inhibitory range of the dynein-ATPase.

These changes in cytoplasmic pH, Na⁺ and Ca²⁺ in sperm from the cauda epididymidis could be responsible for the quiescence state of these cells that let them to conserve energy, which is later required in the female tract. Further experiments are required in order to confirm this hypothesis.

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.

Funding

This research was supported in part by a grant from FONACIT, Venezuela (National Fund of Science and Technology, no. F-2005000222).

Acknowledgements

Dr R D Peralta-Arias was a recipient fellow of a grant from FONACIT (National Fund of Science and Technology), and IMPRES (Venezuelan Medical Federation). The authors thank Dr Stephen Publicover (University of Birmingham, UK) for his important comments on the manuscript. The authors thank Dr Peter Taylor for help in editing the manuscript. They also thank Drs Gustavo Benaim, Fabián Michelangeli, Reinaldo DiPolo and Héctor Rojas for their generous gifts of calmodulin, nigericin, eosin and KB-R7943. F Proverbio and R Marín are co-senior authors of this article.

References

- Afzelius B, Eliasson R, Johnsen Ø & Lindholmer C 1975 Lack of dynein arms in immotile human spermatozoa. *Journal of Cell Biology* **66** 225–232. (doi:10.1083/jcb.66.2.225)
- Aravindan RG, Fomin VP, Naik UP, Modelski MJ, Naik MU, Galileo DS, Duncan RL & Martin-DeLeon PA 2012 CASK interacts with PMCA4b and JAM-A on the mouse sperm flagellum to regulate Ca^{2+} homeostasis and motility. *Journal of Cellular Physiology* **227** 3138–3150. (doi:10.1002/jcp.24000)
- Blanco G, Sanchez G, Melton R, Tourtellote W & Mercer R 2000 The α isoform of the Na,K-ATPase is expressed in the germ cells of the testes. *Journal of Histochemistry and Cytochemistry* **48** 1023–1032. (doi:10.1177/002215540004800801)
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254. (doi:10.1016/0003-2697(76)90527-3)
- Carrasquel G, Camejo M, Michelangeli F & Ruiz M 2009 Relationship between intracellular pH, intracellular $[\text{Ca}^{2+}]$ and sperm motility, 9th International Congress of Andrology. Barcelona, Spain.
- Di Leva F, Domi T, Fedrizzi L, Lim D & Carafoli E 2008 The plasma membrane Ca^{2+} ATPase of animal cells: structure, function and regulation. *Archives of Biochemistry and Biophysics* **476** 65–74. (doi:10.1016/j.abb.2008.02.026)
- Garcia M & Meizel S 1999 Regulation of intracellular pH in capacitated human spermatozoa by a Na^+/H^+ exchanger. *Molecular Reproduction and Development* **52** 189–195. (doi:10.1002/(SICI)1098-2795(199902)52:2<189::AID-MRD10>3.0.CO;2-D)
- Gatto C & Milanick MA 1993 Inhibition of the red blood cell calcium pump by eosin and other fluorescein analogues. *American Journal of Physiology* **264** C1577–C1586.
- Gibbons IR 1965 Chemical dissection of cilia. *Archives de Biologie* **76** 317–352.
- Gibbons I 1974 Mechanisms of flagellar motility. In *The Functional Anatomy of the Spermatozoon*, pp 127–140. Proceedings of the second international symposium, Wenner-Gren Center, Stockholm, August 1973 (Wenner-Gren Center international symposium). Ed. BA Afzelius. Oxford: Pergamon Press.
- Gibbons BH & Gibbons IR 1987 Vanadate-sensitized cleavage of dynein heavy chains by 365-nm irradiation of demembrated sperm flagella and its effect on the flagellar motility. *Journal of Biological Chemistry* **262** 8354–8359.
- Giroux-Widemann V, Pignot-Paintrand I, Jouannet P & Feneux D 1991 The effect of pH on the reactivation of human spermatozoa extracted with Triton X-100. *Molecular Reproduction and Development* **29** 157–162. (doi:10.1002/mrd.1080290211)
- Goodenough UW & Heuser JE 1985 Outer and inner dynein arms of cilia and flagella. *Cell* **41** 341–342. (doi:10.1016/S0092-8674(85)80003-9)
- Hamamah S & Gatti J 1998 Role of the ionic environment and internal pH on sperm activity. *Human Reproduction* **13** 20–30. (doi:10.1093/humrep/13.suppl.4.20)
- Hlivko JT, Chakraborty S, Hlivko TJ, Sengupta A & James PF 2006 The human Na,K-ATPase α 4 isoform is a ouabain-sensitive α isoform that is expressed in sperm. *Molecular Reproduction and Development* **73** 101–115. (doi:10.1002/mrd.20383)
- Jimenez-Gonzalez C, Michelangeli F, Harper CV, Barrat CL & Publicover SJ 2006 Calcium signalling in human spermatozoa: a specialized “toolkit” of channels, transporters and stores. *Human Reproduction Update* **12** 253–267. (doi:10.1093/humupd/dmi050)
- Jouannet P, Escalier D, Serres C & David G 1983 Motility of human sperm without outer dynein arms. *Journal of Submicroscopic Cytology* **15** 67–71.
- Kamiya R, Kurimoto E, Sakakibara H & Okagaki T 1989 A genetic approach to the function of inner and outer arm dynein. In *Cell Movement*, pp 209–218. Eds FD Warner, P Satir & IR Gibbons. New York: Alan R. Liss.
- Kaplan JH 2002 Biochemistry of the Na,K-ATPase. *Annual Review of Biochemistry* **71** 511–535. (doi:10.1146/annurev.biochem.71.102201.141218)
- Kennedy B & Mangini N 1996 Plasma membrane calcium-ATPase in cultured human retinal pigment epithelium. *Experimental Eye Research* **63** 547–556. (doi:10.1006/exer.1996.0145)
- Keskes L, Giroux-Widemann V, Serres C, Pignot-Paintrand I, Jouannet P & Feneux D 1998 The reactivation of demembrated human spermatozoa lacking outer dynein arms is independent of pH. *Molecular Reproduction and Development* **49** 416–425. (doi:10.1002/(SICI)1098-2795(199804)49:4<416::AID-MRD9>3.0.CO;2-Q)
- Koçak-Toker N, Aktan G & Aykaç-Toker G 2002 The role of Na,K-ATPase in human sperm motility. *International Journal of Andrology* **25** 180–185. (doi:10.1046/j.1365-2605.2002.00346.x)
- Krasznai Z, Krasznai Z, Morisawa M, Bazsáné Z, Hernádi Z, Fazekas Z, Trón L, Goda K & Márián T 2006 Role of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in calcium homeostasis and human sperm motility regulation. *Cell Motility and the Cytoskeleton* **63** 66–76. (doi:10.1002/cm.20108)
- Lawson C, Dorval V, Goupil S & Leclerc P 2007 Identification and localisation of SERCA 2 isoforms in mammalian sperm. *Molecular Human Reproduction* **13** 307–316. (doi:10.1093/molehr/gam012)
- Lindemann CB 2003 Structural-functional relationships of the dynein, spokes, and central-pair projections predicted from an analysis of the forces acting within a flagellum. *Biophysical Journal* **84** 4115–4126. (doi:10.1016/S0006-3495(03)75136-4)
- Lindemann CB & Kanous KS 1989 Regulation of mammalian sperm motility. *Archives of Andrology* **23** 1–22. (doi:10.3109/01485018908986783)
- Lingrel J & Kuntzeweiler K 1994 Na^+/K^+ -ATPase. *Journal of Biological Chemistry* **269** 19659–19662.
- Marín R, Proverbio T & Proverbio F 1986 Inside-out basolateral plasma membrane vesicles from rat kidney proximal tubular cells. *Biochimica et Biophysica Acta* **858** 195–201. (doi:10.1016/0005-2736(86)90306-8)
- Marin-Briggiler C, Jha K, Chertihin O, Buffone M, Herr J, Vazquez-Levin M & Visconti P 2005 Evidence of the presence of calcium/calmodulin-dependent protein kinase IV in human sperm and its involvement in motility regulation. *Journal of Cell Science* **118** 2013–2022. (doi:10.1242/jcs.02326)
- Mohamed TM, Zakeri SA, Baudoin F, Wolf M, Oceandy D, Cartwright EJ, Gul S & Neyses L 2013 Optimisation and validation of a high throughput screening compatible assay to identify inhibitors of the plasma membrane calcium ATPase pump – a novel therapeutic target for contraception and malaria. *Journal of Pharmacy & Pharmaceutical Sciences* **16** 217–230.
- Neesen J, Kirschner R, Ochs M, Schmiedl A, Habermann B, Mueller C, Holstein AF, Nuesslein T, Adham I & Engel W 2001 Disruption of an inner arm dynein heavy chain gene results in asthenozoospermia and reduced ciliary beat frequency. *Human Molecular Genetics* **10** 1117–1128. (doi:10.1093/hmg/10.11.1117)
- Okunade GW, Miller ML, Pyne GJ, Sutliff RL, O'Connor KT, Neumann JC, Andringa A, Miller DA, Prasad V, Doetschman T *et al.* 2004 Targeted ablation of plasma membrane Ca^{2+} -ATPase (PMCA) 1 and 4 indicates a major housekeeping function for PMCA1 and a critical role in hyperactivated sperm motility and male fertility for PMCA4. *Journal of Biological Chemistry* **279** 33742–33750. (doi:10.1074/jbc.M404628200)
- Olson GE, Winfrey VP, Winer MA & Davenport GR 1987 Outer acrosomal membrane of guinea pig spermatozoa: isolation and structural characterization. *Gamete Research* **17** 77–94. (doi:10.1002/mrd.1120170109)
- Patel R, Al-Dossary AA, Stabley DL, Barone C, Galileo DS, Strehler EE & Martin-DeLeon PA 2013 Plasma membrane Ca^{2+} -ATPase 4 in murine

- epididymis: secretion of splice variants in the luminal fluid and a role in sperm maturation. *Biology of Reproduction* **89** 6. (doi:10.1095/biolreprod.113.108712)
- Piperno G, Mead K & Shestak W 1992 The inner dynein arms I2 interact with a "dynein regulatory complex" in *Chlamydomonas* flagella. *Journal of Cell Biology* **118** 1455–1463. (doi:10.1083/jcb.118.6.1455)
- Proverbio F, Proverbio T & Marín R 1986 Na⁺-ATPase is a different entity from the (Na⁺+K⁺)-ATPase in rat kidney basolateral plasma membranes. *Biochimica et Biophysica Acta* **858** 202–205. (doi:10.1016/0005-2736(86)90307-X)
- Salvador JM, Inesi G, Rigaud JL & Mata AM 1998 Ca²⁺ transport by reconstituted synaptosomal ATPase is associated with H⁺ countertransport and net charge displacement. *Journal of Biological Chemistry* **273** 18230–18234. (doi:10.1074/jbc.273.29.18230)
- Sanchez G, Nguyen AN, Timmerberg B, Tash JS & Blanco G 2006 The Na,K-ATPase α 4 isoform from humans has distinct enzymatic properties and is important for sperm motility. *Molecular Human Reproduction* **12** 565–576. (doi:10.1093/molehr/gal062)
- Schuh K, Cartwright EJ, Jankevics E, Bundschu K, Liebermann J, Williams JC, Armesilla A, Emerson M, Oceandy D, Knobloch KP *et al.* 2004 Plasma membrane Ca²⁺ ATPase 4 is required for sperm motility and male fertility. *Journal of Biological Chemistry* **279** 28220–28226. (doi:10.1074/jbc.M312599200)
- Shpetner HS, Paschal BM & Vallee RB 1988 Characterization of the microtubule-activated ATPase of brain cytoplasmic dynein (MAP1C). *Journal of Cell Biology* **107** 1001–1009. (doi:10.1083/jcb.107.3.1001)
- Tash JS & Bracho GE 1994 Regulation of sperm motility: emerging evidence for a major role for protein phosphatases. *Journal of Andrology* **15** 505–509. (doi:10.1002/j.1939-4640.1994.tb00489.x)
- Verma R 2001 Sperm quiescence in cauda epididymis: a mini-review. *Asian Journal of Andrology* **3** 181–183.
- Vijayaraghavan S & Hoskins DD 1990 Changes in the mitochondrial calcium influx and efflux properties are responsible for the decline in sperm calcium during epididymal maturation. *Molecular Reproduction and Development* **25** 186–194. (doi:10.1002/mrd.1080250212)
- Vívenes CY, Peralta-Arias RD, Camejo MI, Guerrero K, Fernandez VH, Pinero S, Proverbio T, Proverbio F & Marín R 2009 Biochemical identification of dynein-ATPase activity in human sperm. *Zeitschrift für Naturforschung. Teil C* **64** 747–753.
- Wennemuth G, Babcock DF & Hille B 2003 Calcium clearance mechanisms of mouse sperm. *Journal of General Physiology* **122** 115–128. (doi:10.1085/jgp.200308839)
- World Health Organisation 2010 WHO laboratory manual for the examination and processing of human semen, 5th edn. Geneva, Switzerland: WHO Press, World Health Organisation.
- Williams KM & Ford WC 2003 Effects of Ca-ATPase inhibitors on the intracellular calcium activity and motility of human spermatozoa. *International Journal of Andrology* **26** 366–375. (doi:10.1111/j.1365-2605.2003.00438.x)
- Wong P, Au C & Nigai H 1978 Electrolyte and water transport in rat epididymis, its possible role in sperm maturation. *International Journal of Andrology* **1** (Suppl 2) 608–628. (doi:10.1111/j.1365-2605.1978.tb00512.x)
- Wong P, Lee W & Tsang A 1981 The effects of extracellular sodium on acid release and motility initiation in rat caudal epididymal spermatozoa *in vitro*. *Experimental Cell Research* **131** 97–104. (doi:10.1016/0014-4827(81)90410-9)
- Woo A, James P & Lingrel J 2002 Roles of the Na,K-ATPase α 4 isoform and the Na⁺/H⁺ exchanger in sperm motility. *Molecular Reproduction and Development* **62** 348–356. (doi:10.1002/mrd.90002)
- Yanagimachi R & Usui N 1974 Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. *Experimental Cell Research* **89** 161–174. (doi:10.1016/0014-4827(74)90199-2)
- Yeung CH & Cooper TG 2002 Acquisition and development of sperm motility upon maturation in the epididymis. In *The Epididymis: From Molecules to Clinical Practice*, pp 417–434. Eds B Robaire & B Hinton. New York: Kluwer Academic/Plenum Publishers.
- Young LG & Nelson L 1974 Calcium ions and control of the motility of sea urchin spermatozoa. *Journal of Reproduction and Fertility* **41** 371–378. (doi:10.1530/jrf.0.0410371)

Received 8 September 2014

First decision 8 October 2014

Revised manuscript received 23 February 2015

Accepted 27 February 2015