ATPases, ion exchangers and human sperm motility

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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 Ki(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 (α1 and α4), being α4 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.


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 (Marin-Briggiler et al. 2005). Motility could be modulated by many factors such as intracellular pH (Giroux-Widemann et al. 1991, Garcia & 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 α1 and α4 isoforms have been identified in the midpiece of the sperm, being α4 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 α4 is that it is much more sensitive to ouabain than the α1 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 α4 is sufficient to significantly decrease sperm motility (Koçak-Toker et al. 2002, Woo et al. 2002, Sanchez et al. 2006).
NKA generates a $\text{Na}^+$ gradient across the plasma membrane, providing the energy required for the normal function of the NHE (Wong et al. 1981). NHE exchanges $\text{H}^+$ from the cell for extracellular $\text{Na}^+$, 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 $\text{Ca}^{2+}$ pump (PMCA) is an ATPase that extrudes $\text{Ca}^{2+}$ from the cell (Salvador et al. 1998), and is involved in the fine control of the intracellular $\text{Ca}^{2+}$ concentration. ATP2B4 is the major isof orm 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 $\text{Ca}^{2+}$, a resting value of $1.4 \times 10^{-7} \text{ M}$ up to $1.28 \times 10^{-6} \text{ M}$ (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 (berpridil, DCB and KB-R7943) leads to a rise in intracellular $\text{Ca}^{2+}$ 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 (Aifenius 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 (Vivesenes 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 $\text{Ca}^{2+}$ on the dynein-ATPase activity of human sperm. Together, these data contribute to understand the relationship between pH, $\text{Ca}^{2+}$ 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 in 0.5 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 Olsson et al. (1987) and Vivesenes 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 benzamidine, 1 μg/ml leupeptin and 1 μg/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 11 132 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. For ATPase activity. Protein was determined by the Bio-Rad micro method (Bio-Rad Laboratories) based on Bradford’s assay (Bradford 1976).

**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) 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 amounts of phosphate liberated in the test tubes in the presence and absence of ouabain.

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 (Viñenes et al. 2009).

**Statistical analysis**

The results are expressed as mean±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 Dunnet’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 z1 and z4 isoforms. The z4 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 z4 isoform is much more sensitive to ouabain than z1, 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 z4 isoform of the NKA is much more sensitive to ouabain than z1, both in humans and rodents (Woo et al. 2002, 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 z1 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±3% forward progressives, control vs 48±4% forward progressives, 1×10^{-6} M ouabain: n=3, P<0.01). In other words, the inhibition of the z4 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
Each component was analysed separately, giving two components with different sensitivity to the drug. 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. 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ remain largely unknown. In the current study, we used eosin, a known inhibitor of ATP2B4 (Gatto & Milanick 1993, Kennedy & Mangini 1996, Mohamed et al. 2006) 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 ± 2% at 180 min of incubation, P<0.001) or 2.5 × 10⁻⁵ M KB-R7943 (37 ± 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 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 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).

**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

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**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 × 10⁻⁴ 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 ± S.E.M. The multiple comparisons for the same incubation time were performed with Dunnet’s test.

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

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<tr>
<th>Incubation medium</th>
<th>ATPase activity (nmol Pi/mg prot. min)</th>
<th>NKA</th>
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<tr>
<td>(a) Mg²⁺ + Na⁺ + ouabain</td>
<td>272 ± 8</td>
<td>99 ± 6* (b and a)</td>
</tr>
<tr>
<td>(b) Mg²⁺ + Na⁺ + K⁺</td>
<td>371 ± 12</td>
<td>97 ± 7* (b and c)</td>
</tr>
<tr>
<td>(c) Mg²⁺ + Na⁺ + K⁺ + ouabain</td>
<td>274 ± 9</td>
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Assays made according to the ‘Materials and methods’ section. The ouabain concentration used was 2 × 10⁻⁸ M. Activity values expressed as mean ± S.E.M., n = 12. The S.E.M. for the NKA activity was calculated for paired data (*P<0.001).
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\(^{-1}\) 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.

**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

![Dynein-ATPase activity and calcium](image)

**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\(\times\)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 ± S.E.M. of quadruplicate determinations from 20 separate experiments with different preparations.

**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.
reversed in the presence of norepinephrine (Viveses 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 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 ± S.E.M. The multiple comparisons for the same incubation time were performed with Dunnet’s test. *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 ± S.E.M. The multiple comparisons for the same incubation time were performed with Dunnet’s test. *P < 0.001 vs control.

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 ± S.E.M. The multiple comparisons for the same incubation time were performed with Dunnet’s test. *P < 0.001 vs control and **P < 0.001 vs control, vs 1 × 10$^{-4}$ M ouabain and vs 2.5 × 10$^{-5}$ M KB-R7943. (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 ± S.E.M. The multiple comparisons for the same incubation time were performed with Dunnet’s test. *P < 0.001 vs control and **P < 0.001 vs control, vs 1 × 10$^{-4}$ M ouabain and vs 2.5 × 10$^{-5}$ M KB-R7943.
VSL (VCL (G account the well-documented importance of Ca2+ in participating in the regulation of motility. Taking into account 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, Sanchez 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 α1 and α4 isoforms of NKA are expressed in the midpiece of human sperm (Hlivko et al. 2006), and selective inhibition of α4 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 Ca2+ 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 Ca2+ 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 Ca2+ 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 Ca2+, 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 [Ca2+] 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 axoneme-containing fraction of human sperm (Carrasquel et al. 2009). The dynein-ATPase activity of 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 MgCl2; 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. *P < 0.01 vs 0 free [Ca2+] and †P < 0.01 vs 0 free [Ca2+] (Fig. 6).

<table>
<thead>
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<th>CASA parameter</th>
<th>Incubation condition</th>
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<tr>
<td></td>
<td>Control</td>
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<tr>
<td>% FP</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>VCL (μm/s)</td>
<td>96 ± 4</td>
</tr>
<tr>
<td>VSL (μm/s)</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>VAP (μm/s)</td>
<td>45 ± 2</td>
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<tr>
<td>BCF (beats/s)</td>
<td>15 ± 1</td>
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<tr>
<td>ALH (μm)</td>
<td>4.0 ± 0.2</td>
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</table>

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^{-6} M amiloride and 2.5 × 10^{-6} M KB-R7943. The CASA parameters related to sperm motility were 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.

Table 2 Comparison of the effects of several inhibitors on the kinematic parameters in human semen.
axoneme (Lindemann 2003). This is regulated by phosphorylation–dephosphorylation processes in the outer dynein arm (ODA), through AMP–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 Ca^{2+} 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 (Viveneves 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 ODA's (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 demembranated and incubated with appropriate concentrations of Mg^{2+}-ATP. The reactivation profile of LODA spermatozoa as a function of Mg^{2+}-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 Mg^{2+}-ATP and removal of the free Ca^{2+} 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 ‘Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} in sperm from the caput epididymis 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 Ca^{2+} 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 Ca^{2+} in an inhibitory range of the dynein-ATPase.

These changes in cytoplasmic pH, Na^{+} and Ca^{2+} in sperm from the cauda epididymis 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.

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