Evidence of the presence of calcium/calmodulin-dependent protein kinase IV in human sperm and its involvement in motility regulation

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Summary

The mechanisms involved in the regulation of mammalian sperm motility are not well understood. Calcium ions (Ca^{2+}) have been suggested to play a key role in the maintenance of motility; nevertheless, how Ca^{2+} modulates this process has not yet been completely characterized. Ca^{2+} can bind to calmodulin and this complex regulates the activity of multiple enzymes, including Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases). Results from this study confirmed that the presence of Ca^{2+} in the incubation medium is essential for maintaining human sperm motility. The involvement of CaM kinases in Ca^{2+} regulation of human sperm motility was evaluated using specific inhibitors (KN62 and KN93) or their inactive analogues (KN04 and KN92 respectively). Sperm incubation in the presence of KN62 or KN93 led to a progressive decrease in the percentage of motile cells; in particular, incubation with KN62 also reduced sperm motility parameters. These inhibitors did not alter sperm viability, protein tyrosine phosphorylation or the follicular fluid-induced acrosome reaction; however, KN62 decreased the total amount of ATP in human sperm. Immunological studies showed that Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) is present and localizes to the human sperm flagellum. Moreover, CaMKIV activity increases during capacitation and is inhibited in the presence of KN62. This report is the first to demonstrate the presence of CaMKIV in mammalian sperm and suggests the involvement of this kinase in the regulation of human sperm motility.

Key words: Human sperm, Calcium, Calmodulin, CaM kinases, Motility, Capacitation.

Introduction

After leaving the testis, mammalian spermatozoa are morphologically differentiated but are immotile and unable to fertilize. Progressive motility is acquired during epididymal transit and fertilization capacity is gained while sperm traverse the female reproductive tract in a process called capacitation. Capacitation involves several changes, occurring in both the sperm head and tail that lead to the release of the acrosomal content (acrosome reaction, AR) and to the acquisition of a distinct type of motility known as hyperactivation. Both events are essential for sperm penetration through the egg coatings (Yanagimachi, 1994).

The molecular basis of capacitation is not well understood. Results from several laboratories have shown that capacitation is associated with a phosphorylation cascade. In particular, several proteins undergo a cyclic adenosine monophosphate (cAMP)-dependent increase in tyrosine phosphorylation (Visconti et al., 1995a; Visconti et al., 1995b). Many of these proteins are Triton X-100 insoluble, localize to the principal piece of the flagellum, and have been proposed to be involved in the regulation of sperm motility (Carrera et al., 1996). In this respect sperm tail proteins, A kinase-anchoring proteins (AKAP) 3 and 4, undergo capacitation-dependent phosphorylation in both tyrosine and serine residues (Carrera et al., 1996; Ficarro et al., 2003; Mandal et al., 1999), suggesting that both tyrosine and serine/threonine kinases participate in sperm motility regulation.

In addition to the role of phosphorylation signalling in sperm motility, studies with a variety of species have shown that Ca^{2+} ions play a key role in the development and maintenance of sperm progressive motion and hyperactivation (Lindemann et al., 1991; Serres et al., 1991; Marin-Briggiler et al., 1999; Marin-Briggiler et al., 2003; Marquez and Suarez, 2004). Using demembranated sperm models, some of these investigations have revealed that Ca^{2+} levels modulate the waveform of the sperm tail (Ho et al., 2002; Lindemann and Goltz, 1988). However, the underlying Ca^{2+}-regulated pathways involved in the initiation and maintenance of sperm motility have not yet been fully characterized. Ca^{2+} can directly bind to membrane phospholipids and to several enzymes, with the subsequent modification of membrane properties and enzymatic activity. This cation may also act through binding

to calmodulin (CaM); in this regard, a recent study (Si and Olds-Clarke, 2000) demonstrated that CaM antagonists inhibited different aspects of sperm function, including the onset of sperm hyperactivation. Ca^{2+} binding to CaM changes its conformation and the Ca^{2+}/CaM complex regulates the activity of multiple enzymes, including adenylyl cyclases (Gross et al., 1987; Mons et al., 1999), phosphatases (Rusnak and Mertz, 2000; Tash et al., 1988), phosphodiesterases (Wasco and Orr, 1984) and protein kinases (Hook and Means, 2001). Most of these enzymes are present in mammalian sperm and appear to be involved in the regulation of flagellar bending (Turner, 2003). Recently, Zeng and Tulsiani (Zeng and Tulsiani, 2003) demonstrated that CaM has a role in sperm capacitation. These authors used different CaM antagonists and showed that although some affected motility without altering sperm viability, all of them inhibited the agonist-induced acrosome reaction. These results strongly suggest that CaM antagonists can prevent capacitation by interfering with multiple regulatory pathways. Consistent with these results are the aforementioned multiple targets for CaM action.

Kinases regulated by Ca^{2+}/CaM belong to a subfamily known as CaM kinases (CaMK) comprised of CaMKI, II and IV. CaM kinases are serine/threonine kinases that phosphorylate transcription factors and thus, play a major role in Ca^{2+}-regulated gene expression in somatic cells. CaM kinases have limited tissue distribution; presence of these enzymes has been reported in brain, thymus, bone marrow, ovary and testis (Means et al., 1991). Several reports indicate that CaMKII is expressed in oocytes and is activated during fertilization, mediating meiosis resumption and cortical granule exocytosis (Johnson et al., 1998; Lorca et al., 1993). Regarding the testis, CaMKIV has been localized to mouse spermatogonia and spermatids. In elongating spermatids (transcriptionally inactive cells), CaMKIV is associated with the nuclear matrix and might be involved in chromatin remodelling during nuclear condensation (Wu and Means, 2000). Two recent studies focused on CaMKIV-deficient mice fertility: one reported that these animals are infertile owing to impaired spermiogenesis and replacement of basic nuclear proteins by protamines (Wu et al., 2000a), the other found that they exhibited normal spermatogenesis and fertility (Blaeser et al., 2001). The presence and role of these enzymes in sperm have not yet been described.

In this work, we have attempted to examine the potential mechanisms involved in the Ca^{2+} regulation of human sperm motility. Evidence is presented showing that incubation of human sperm with inhibitors of the CaM kinase family (KN62 and KN93), but not with their inactive analogues (KN04 and KN92, respectively), results in a significant decrease of sperm motility and ATP content. In addition, we show that CaMKIV is present in mammalian sperm and localizes to the flagellum.

Materials and Methods

Culture media

Modified Tyrode's medium, HSM (Suarez et al., 1986), was used throughout the study. It consisted of 117.5 mM NaCl, 0.3 mM NaH_2PO_4, 8.6 mM KCl, 0.49 mM $MgCl_2$, 2 mM glucose, 19 mM sodium lactate, 25 mM $NaHCO_3$, 0.25 mM sodium pyruvate, 50 μg/ml penicillin and 75 μg/ml streptomycin. The medium not supplemented with $CaCl_2$ was called '$–Ca^{2+}$'. When this medium was supplemented with 2.6% bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO), it contained 0.1 mM Ca^{2+}, as determined by atomic absorption. These measurements were carried out by Pharma Control laboratory, Buenos Aires, Argentina, using a Shimadzu AA-660 spectrophotometer as described (Association of Official Analytical Chemists, 2000). To prepare media with increasing Ca^{2+} concentrations, different amounts of a stock solution of 22.5 mM $CaCl_2$ were added to $–Ca^{2+}$ +2.6% BSA, giving the following final concentrations of Ca^{2+}: 0.22, 0.58, 1.5 and 2.5 mM. Medium containing maximum amounts of $CaCl_2$ (2.5 mM) was called '$+Ca^{2+}$'. All these media had similar osmolarity (308-314 mOsm).

Semen samples and sperm processing

Semen samples were obtained from normozoospermic volunteers according to WHO standards (WHO, 1999). In each experiment, ejaculates from different men were used. After complete liquefaction, samples were subjected to sperm selection using glass wool columns (Microfibre Manville, Denver, CO) (Calvo et al., 1989) and highly motile cells were resuspended in $–Ca^{2+}$ medium supplemented with 2.6% BSA. In some cases (western blot and CaMKIV activity experiments), sperm separation was carried out using Percoll™ gradients, as described (Ficarro et al., 2003). Sperm concentration was adjusted to 5×10^6 cells/ml and up to 2 ml aliquots were incubated under different conditions at 37°C, 5% CO_2 in air for different periods of time as stated. When indicated, CaM kinase inhibitors KN62 and KN93 (Calbiochem, San Diego, CA) or their inactive analogues KN04 (Seikagaku, Tokyo, Japan) and KN92 (Calbiochem), were added immediately after sperm filtration. These compounds were originally prepared as 20 mM stock solutions in DMSO. In each experiment, an aliquot containing DMSO concentrations equivalent to the maximum concentration of inhibitors used (0.3 to 0.5% depending on the experiment), was included. After a 30-minute incubation, all aliquots were supplemented with 2.5 mM $CaCl_2$, except one that served as control ($–Ca^{2+}$). Post incubation motility was determined by observation under light microscopy (400× magnification; Alphaphot-2 YS2, Nikon, Tokyo, Japan) or by computer-assisted sperm analysis (see below). Sperm viability was assessed using 0.5% Eosin Y in aqueous 0.9% NaCl solution, as indicated (WHO, 1999). In all cases, at least 200 cells were scored.

Computer-assisted sperm analysis (CASA)

Aliquots of 50,000 sperm incubated for 18 hours under different experimental conditions were placed in a MicroCell™ chamber (Conception Technologies, San Diego, CA) and analysed using an IVOS V10.8s CASA instrument (Hamilton-Thorne Research, Beverly, MA). The settings used during the analysis were: number of frames acquired=30, frame rate=60 Hz, minimum contrast=80, minimum cell size=3 pixels, minimum static contrast=30. The kinematic values for at least 200 motile sperm were analysed in each sample.

To determine the percentage of hyperactivated sperm, analysis was performed after a 4-hour incubation under different conditions. Cells with curvilinear velocity (VCL)≥100 μm/second, lateral head displacement (ALH)≥5 μm and linearity (LIN)≤60% were considered hyperactivated (Mortimer and Mortimer, 1990).

Electrophoresis and western blots

Presence of CaM kinases was evaluated in protein extracts from non-capacitated human sperm. Cells were washed twice with PBS, resuspended in sample buffer in the absence of 2-β-mercaptoethanol (Laemmli, 1970), boiled and centrifuged at 6000 ***g*** for 5 minutes. The supernatants were removed and heated at 100°C in the presence of

2-β-mercaptoethanol (5% final concentration) for 5 minutes. Following centrifugation, the supernatants were stored at –20°C until use. Solubilized proteins, corresponding to 2×10^6 sperm (3 μg protein measured using the ABC kit from Pierce) per lane, were separated under reducing conditions by 12% SDS-PAGE (Laemmli, 1970). For two-dimensional (2-D) gel analysis, sperm were solubilized in a lysis buffer (Celis) consisting of 2% (v/v) NP-40, 9.8 M urea, 100 mM dithiothreitol (DTT), 2% (v/v) ampholines (pH 3.5-10), and the protease inhibitors 2 mM PMSF, 5 mM iodoacetamide, 5 mM EDTA, 3 mg/ml L-1chlor-3-(4-tosylamido)-7-amino-2-heptanon-hydrochloride (TLCK), 1.46 μM pepstatin A and 2.1 μM leupeptin. 5×10^8 cells/ml were solubilized by constant shaking at 4°C for 60 minutes. Insoluble material was removed by centrifugation. 65 μl sperm extract (approximately 0.15 mg of protein) were subjected to 2-D gel electrophoresis as described (Ficarro et al., 2003). Electrophoretic transfer was carried out as detailed (Kalab et al., 1994) and membranes were blocked with PBS containing 10% fish gelatin and 0.1% Tween 20 (Sigma). Monoclonal anti-CaMKIV or anti-CaMKII (Transduction Laboratories, Lexington, KY; 1:2000) were used as primary antibodies. Blots were washed four times for 5 minutes, and incubated with anti-mouse peroxidase-conjugated IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) (1:5000).

Tyrosine-phosphorylated proteins were detected in cells incubated for 18 hours under different experimental conditions. Sperm were processed as previously detailed (Marin-Briggiler et al., 1999). Extracts (corresponding to 2×10^6 sperm or 3 μg protein per lane) were subjected to 7% SDS-PAGE, and protein transferred onto nitrocellulose membranes. Loading of equal amounts of protein in each lane was checked by Ponceau staining. Phosphotyrosine residues were detected using a monoclonal antibody (clone 4G10, Upstate Biotechnology, NY; 1:10,000). Blots were washed four times for 5 minutes, and secondary antibody (1:5000) was added for 1 hour. After incubation with the antibodies, membranes were extensively washed and reactive bands were detected by enhanced chemiluminescence using the ECL kit (Amersham Life Science, Oakville, ON) according to the manufacturer's instructions. All incubations were performed at room temperature.

Acrosome reaction (AR) assessment

To determine whether CaM kinases are involved in human sperm AR, CaM kinase inhibitors (60 μM) were added immediately after sperm recovery. 30 minutes later, sperm suspensions were supplemented with 2.5 mM Ca^{2+} and incubated for an additional 18 hours. Aliquots incubated in medium supplemented or not with $CaCl_2$ (+Ca^{2+} and –Ca^{2+}, respectively) served as controls. Induction of the AR was performed at the end of the incubation period by sperm exposure to 10% human follicular fluid (hFF) or buffer for 45 minutes at 37°C, 5% CO_2 in air (Marin-Briggiler et al., 1999). After this procedure, sperm were fixed and the AR evaluated by staining with 50 μg/ml fluorescein isothiocyanate (FITC)-labelled *Pisum sativum* agglutinin (Sigma) as previously described (Marin-Briggiler et al., 1999). Stained cells (at least 200 cells per treatment) were scored in a microscope equipped with epifluorescence (Nikon Labophot, Tokyo, Japan). Presence of a bright staining over the acrosome was indicative of intact sperm; cells with partial or patchy staining, with fluorescence restricted to the equatorial segment or lack of staining were considered acrosome reacted. Under the conditions used in these experiments the predominant patterns were the bright staining over the acrosome or the equatorial segment fluorescence. The patchy staining was restricted to less than 3% of the cells in all the conditions analysed.

Determination of ATP levels

ATP concentration was measured in sperm (10×10^6/ml) incubated for 18 hours under different experimental conditions. Capacitation medium (0.4 ml) was removed by centrifugation, and the sperm pellet was washed twice with PBS. Cells were resuspended in 0.1 ml PBS, placed in glass tubes and 0.4 ml of Tris-acetate buffer (Labsystems, Helsinki, Finland) was added. Samples were heated at 100°C for 15 minutes to release intracellular ATP and stored at –20°C until use. ATP was measured using a kit from Labsystems according to the manufacturer's instruction. Briefly, on the day of the measurement, 0.3 ml Tris-acetate buffer and 0.1 ml ATP monitoring reagent (Labsystems) were mixed in a cuvette and background light emission (*B* value) was recorded in a luminometer (1250, LKB Wallac, Turku, Finland). 0.1 ml of the sample was added, and luminescence was measured (*S* value). After addition of 0.05 nmoles of standard ATP solution (Labsystems), the *I* value was determined. *S* and *I* values were corrected for background luminescence (*Sc* and *Ic*, respectively) and results were calculated as: nmoles ATP per 10^6 cells=$[Sc/(Ic–Sc)]\times 0.05$ nmoles.

Immunofluorescence microscopy

Non-capacitated sperm were air-dried onto glass slides, washed three times with PBS, permeabilized with methanol, washed again with PBS and blocked with 10% normal goat serum (NGS) in PBS. Incubations were then carried out with anti-CaMKIV and anti-CaMKII antibodies (Transduction Laboratories, 1:250) or normal mouse serum (1:250) diluted in PBS with 1% NGS (PBS-NGS). After washing, slides were incubated with FITC-conjugated $F(Ab)_2$ fragments of donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) (1:200) in PBS-NGS, washed with PBS and mounted with Slow-Fade Light (Molecular Probes, Eugene, OR). Sperm were observed by differential interference contrast and epifluorescence microscopy using a Zeiss axiophot microscope (Carl Zeiss, Thornwood, NY).

CaM kinase IV activity

CaMKIV activity was measured in motile sperm treated immediately after Percoll™ selection and resuspension in the incubation medium or in sperm incubated for 18 hours in capacitating conditions in the absence or presence of 60 μM KN62. Sperm subjected to these different experimental conditions were washed twice with PBS, cell concentration was adjusted to 10^7 cells/ml, and aliquots were stored at –20°C until use. After thawing, 10 μl sperm suspension were mixed with an equal amount of 2× reaction buffer, consisting of 25 mM HEPES, 40 μM ATP, 1 μCi/assay [γ-^{32}P]ATP, 10 mM $MgCl_2$, 10 μM aprotinin and leupeptin, 100 μM Na_3VO_4, 5 mM p-NO_2-phenol phosphate, 40 mM glycerophosphate, 1% Triton X-100, 1 mg/ml BSA and 100 μM peptide gamma (Biomol, PA), as specific substrate. After a 15-minute incubation at 37°C, the reaction was stopped with the addition of 20 μl of 20% trichloroacetic acid as described (Visconti et al., 1997). Samples were placed on ice for 20 minutes, centrifuged, and 30 μl supernatant were spotted onto phosphocellulose paper (2×2 cm Whatman p 81). After five washes with 5 mM phosphoric acid, the phosphocellulose papers were dried and placed in vials. 2.5 ml scintillation fluid was added to each vial and counted in a scintillation counter. Assays were run in triplicate and results were expressed as fmoles of ^{32}P incorporated/10^6 sperm/minute.

Statistical analysis

Data were expressed as mean±s.e.m. To assume normal distribution, percentages were converted to ratios, and subjected to the arcsine square root transformation. Data were compared by one- or two-way ANOVA and Student-Newman-Keuls multiple comparison test. Statistical analyses were performed using the GraphPad InStat program (GraphPad Software, San Diego, CA).

Results

Calcium ions are required for the maintenance of human sperm motility in vitro

To determine the requirement of extracellular Ca^{2+} for the maintenance of human sperm motility in vitro, cells were incubated for up to 18 hours in medium not supplemented with $CaCl_2$ ($-Ca^{2+}$: containing 0.1 mM contaminating Ca^{2+} as determined by atomic absorption) or containing increasing concentrations of this cation (0.22, 0.58, 1.5 and 2.5 mM), and the percentage of progressively motile and viable cells were determined. When sperm were incubated in $-Ca^{2+}$ medium there was a significant decline in the percentage of motile cells compared to other conditions (Fig. 1A); such a decrease was not due to cell death (Fig. 1B). In contrast, maximum percentages of motility and viability were obtained in cells incubated in the presence of 0.22 mM or higher Ca^{2+} concentrations (Fig. 1).

CaM kinase inhibitors decrease human sperm motility

The aforementioned results showing that Ca^{2+} are required for the maintenance of human sperm motility, added to the findings that some CaM antagonists inhibit mouse sperm motion (Si and Olds-Clarke, 2000; Zeng and Tulsiani, 2003), suggest that the effect of Ca^{2+} may be mediated through a Ca^{2+}/CaM pathway. This evidence suggests that CaM kinases may also participate in the modulation of mammalian sperm motility. To analyse this possibility, motile human sperm were suspended in medium not supplemented with Ca^{2+} in the presence or absence of CaM kinase inhibitors (KN62 and KN93), or their inactive analogues (KN04 and KN92, respectively). These compounds were added to give the following final concentrations: 1, 3, 10, 30, 60, 100 μM. CaM kinase inhibitors are competitive inhibitors of the activation of CaM kinases by Ca^{2+}/CaM; therefore, to maximize their activity, they were added before supplementation with Ca^{2+}. After a 30-minute incubation, aliquots were supplemented with $CaCl_2$ to a final concentration of 2.5 mM and sperm were incubated for 18 hours in capacitating conditions. Aliquots incubated in $-Ca^{2+}$ medium, in the absence of the inhibitors, or in the presence of the maximum concentration of DMSO served as controls. Overnight incubation with KN62 led to a concentration-dependent and significant reduction in the percentage of motile cells (Fig. 2A). These percentages were similar to those obtained in medium not supplemented with Ca^{2+}. Incubation with increasing concentrations of KN93 also produced a decrease in sperm motility, although the decline was not as pronounced as that observed with KN62. The effect of CaM kinase inhibitors upon sperm motion was specific, as no decrease in the percentage of motile sperm was observed in the presence of similar concentrations of the inactive analogues of the CaM kinase inhibitors or in the presence of DMSO (Fig. 2A). The percentage of viable cells was similar in all conditions (Fig. 2B).

To further investigate the involvement of CaM kinases in the development and maintenance of human sperm motility, cells were incubated in the presence of KN62 or KN04 (60 μM) as previously described, and the percentage of motile and live cells at different time points was determined. The results revealed that KN62 causes a significant and progressive decline in the percentage of motile sperm (Fig. 3A). The values obtained in the presence of 60 μM KN62 were similar to those observed in cells incubated for the same period of time in the $-Ca^{2+}$ medium. In all the conditions tested, sperm remained viable throughout the incubation period (Fig. 3B).

The effect of KN62 upon sperm motility was also studied using computer-assisted sperm analysis (CASA) of motion parameters. Experiments were conducted after an 18-hour incubation in $-Ca^{2+}$ medium, and in the presence of Ca^{2+} with or without the addition of 60 μM KN62 or its inactive analogue KN04 (Table 1). When incubations were carried out in the presence of KN62 or in $-Ca^{2+}$ medium, there was a reduction in the percentage of progressive cells, in the average path velocity (VAP), in the straight-line velocity (VSL) and in the percentage of cells displaying rapid motion (Grade 4); the decrease in these parameters observed in the presence of KN62 was similar to that observed in $-Ca^{2+}$. Confirming the results shown (Figs 1-3), human sperm incubated in the presence of KN62 or in medium not supplemented with Ca^{2+} showed a significant increase in the percentage of static sperm (Grade 1). In cells incubated in both conditions, other sperm motility parameters such as the curvilinear velocity (VCL), the amplitude of lateral head displacement (ALH), the beat/cross frequency (BCF), the percentage of straightness (STR) and the percentage of linearity (LIN) were slightly decreased, but they did not show significant differences when compared to controls.

Several reports indicate that the cyclic adenosine

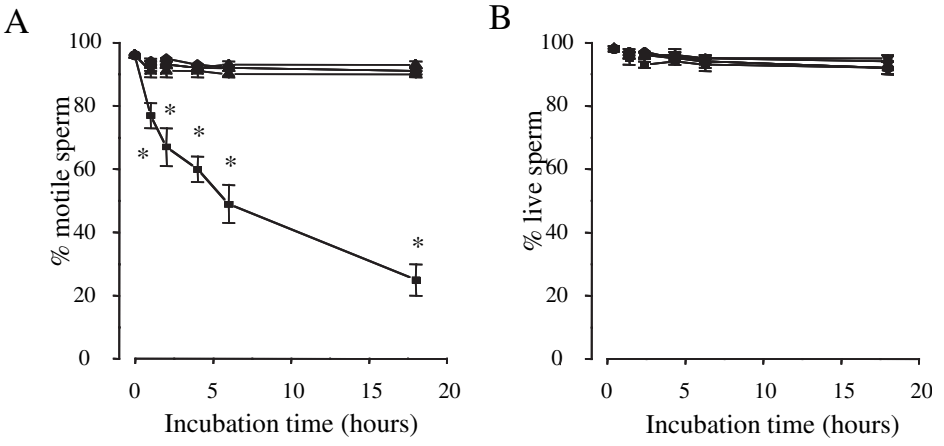

Fig. 1. Ca^{2+} requirement for the maintenance of human sperm motility and viability. Motile sperm selected using glass wool columns were incubated for up to 18 hours at 37°C in medium not supplemented with $CaCl_2$ ($-Ca^{2+}$, ■), or containing different concentrations of $CaCl_2$: 0.22 mM (▲), 0.58 mM (▼), 1.5 mM (◆) or 2.5 mM (●). The percentages of progressively motile (A) and live (B) cells after 1, 2, 4, 6 and 18-hour incubation were determined as described in Materials and Methods. The results are expressed as mean±s.e.m., n=7. *P<0.05 when compared to sperm motility under all other conditions.

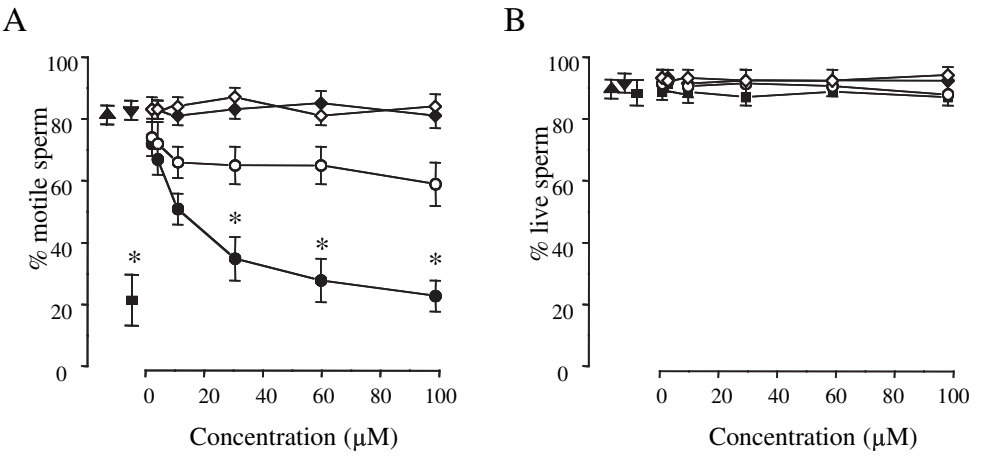

Fig. 2. Concentration curve of CaM kinase inhibitors effect upon human sperm motility and viability. Immediately after selection using glass wool columns, motile sperm were resuspended in medium not supplemented with $CaCl_2$, and in the presence or absence of the CaM kinase inhibitors KN62 (●) or KN93 (○); or their inactive analogues KN04 (◆) and KN92 (◇) at the following final concentrations: 1, 3, 10, 30, 60, 100 μM. After 30 minutes, aliquots were supplemented with $CaCl_2$ (final concentration: 2.5 mM) and cells were incubated for 18 hours in capacitating conditions. Aliquots incubated in medium not supplemented with $CaCl_2$ (– Ca^{2+}, ■), in the absence of the inhibitors (+ Ca^{2+}, ▼), or in the presence of 0.5% DMSO (DMSO, ▲) served as controls. The percentages of progressively motile (A) and live (B) cells were determined. The results are expressed as mean±s.e.m., n=6. *P<0.01 when compared to sperm motility under all other conditions.

monophosphate/protein kinase A (cAMP/PKA) pathway is involved in the regulation of flagellar bending (Eddy and O'Brien, 1994; Luconi and Baldi, 2003). To determine the relationship between this pathway and that involving CaM kinases, 1 mM dibutyryl cAMP (a cAMP agonist) and 200 μM isobutyl-methylxanthine (a cAMP phosphodiesterase inhibitor) were simultaneously added with KN62 (60 μM), and the effect upon sperm motion was assessed. The addition of these compounds could not restore motility in cells incubated with KN62 for 18 hours (data not shown), suggesting that CaM kinases participate in motility regulation downstream to or independently from a cAMP/PKA pathway.

Because previous works in sperm from different species (Marin-Briggiler et al., 2003; Marquez and Suarez, 2004) have shown that Ca^{2+} is required for the development of hyperactivated motility, the possibility that CaM kinases are involved in the regulation of hyperactivation was also analysed. Hyperactivation parameters were investigated as described in human sperm incubated for 4 hours under capacitating conditions, in media supplemented or not with Ca^{2+} and in the presence or in the absence of 60 μM KN62 or its inactive analogue KN04. In Ca^{2+}-containing medium (2.5 mM), the percentage of hyperactivated cells was 19±3%. As reported (Marin-Briggiler et al., 2003), the development of this type of motility was significantly inhibited in –Ca^{2+} medium (3±1%). Although incubation with KN62 reduced the percentage of cells displaying hyperactivation to 10±2%, a similar reduction was observed in the presence of KN04 (8±1%), suggesting a non-specific effect of these compounds on hyperactivation.

CaM kinase inhibitors do not inhibit the Ca^{2+}-induced decrease in tyrosine phosphorylation or the hFF-induced acrosome reaction

The development of mammalian sperm motility and the occurrence of capacitation has been related to phosphorylation of several sperm proteins on tyrosine residues (Osheroff et al., 1999; Visconti et al., 2002). In human sperm, as well as in mouse sperm under certain conditions, the presence of Ca^{2+} in the incubation medium decreases protein tyrosine phosphorylation levels (Carrera et al., 1996; Luconi et al., 1996; Marin-Briggiler et al., 2003; Baker et al., 2004). CaM

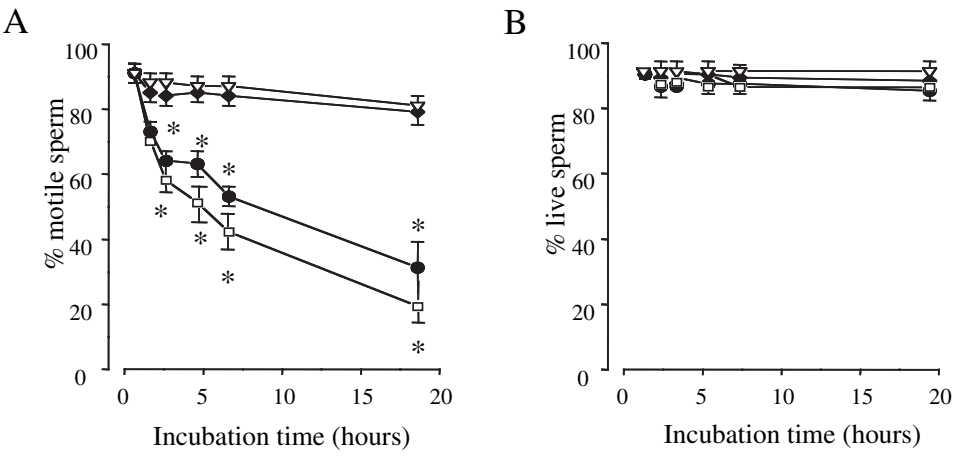

Fig. 3. Time curve of the effect of CaM kinase inhibitor (KN62) and its inactive analogue (KN04) upon human sperm motility and viability. Immediately after selection with glass wool columns, motile sperm were resuspended in medium not supplemented with $CaCl_2$, and 60 μM KN62 (●) or KN04 (◆) were added. After 30 minutes, aliquots were supplemented with $CaCl_2$ (final concentration: 2.5 mM) and cells were incubated for up to 18 hours in capacitating conditions. Aliquots incubated in medium not supplemented with $CaCl_2$ (–Ca^{2+}, □) and in the absence of the inhibitors (+Ca^{2+}, ▽) served as controls. The percentages of progressively motile (A) and live (B) cells after 1, 2, 4, 6 and 18-hour incubations were determined. The results are expressed as mean±s.e.m., n=7. *P<0.01 when compared with sperm motility under control conditions.

Table 1. Kinematic characteristics of human sperm incubated with a CaM kinase inhibitor or its inactive analogue

	$-Ca^{2+}$	KN62	KN04	$+Ca^{2+}$
Total motility (%)	15±6**	26±9**	66±5	74±2
Progressive (%)	5±4*	13±7*	42±10	56±6
VAP	33±3*	37±5*	47±7	54±4
VSL	24±4*	30±6	41±8	48±5
VCL	66±8	64±6	75±6	84±4
ALH	2±1	4±1	4±1	4±1
BCF	23±6	22±1	22±1	26±4
STR	70±5	78±3	84±5	87±3
LIN	38±3	45±4	52±7	56±5
Grade 4 (%)	13±7*	19±8*	54±7	68±2
Grade 3 (%)	2±1*	7±2	12±2	7±1
Grade 2 (%)	7±3	6±4	12±1	10±3
Grade 1 (%)	78±10**	68±13**	22±6	16±4

Immediately after selection using glass wool columns, motile sperm were resuspended in medium not supplemented with $CaCl_2$, and 60 μM KN62 or KN04 were added. After 30 minutes, aliquots were supplemented with $CaCl_2$ (final concentration: 2.5 mM) and cells were incubated for up to 18 hours in capacitating conditions. Aliquots incubated in medium not supplemented with $CaCl_2$ ($-Ca^{2+}$) and in the absence of the inhibitor or the inactive analogue ($+Ca^{2+}$) served as controls. Parameters measured: average path velocity (VAP, μm/second), straight-line velocity (VSL, μm/second), curvilinear velocity (VCL, μm/second), amplitude of lateral head displacement (ALH, μm), beat/cross frequency (BCF, Hz), straightness (STR,%), linearity (LIN,%). According to their motility, individual sperm were classified as Grade 4 (rapid motion, VAP >25 μm/second), Grade 3 (slow motion, VAP 25-5 μm/second), Grade 2 (in situ motility, VAP <5 μm/second), Grade 1 (static cells). The results are expressed as mean±s.e.m., n=3.

*P<0.05, **P<0.01 when compared to values in KN04 and $+Ca^{2+}$ treated cells.

antagonists have been shown to inhibit this Ca^{2+}-induced decrease in protein tyrosine phosphorylation (Carrera et al., 1996). In the present report we explored whether Ca^{2+} effect was linked to the activation of CaM kinases. Motile sperm were treated as previously indicated, and protein tyrosine phosphorylation patterns were analysed by western blotting using an anti phosphotyrosine antibody. As expected, sperm incubated in $-Ca^{2+}$ medium showed higher levels of protein tyrosine phosphorylation than those incubated in the presence of this divalent cation ($+Ca^{2+}$). Similar low levels of protein tyrosine phosphorylation were obtained either in the presence of KN62, KN04 (60 μM), DMSO (Fig. 4A,B), KN93 or KN92 (data not shown). These results suggest that in human sperm, CaM kinases do not participate in the Ca^{2+}-associated decrease in protein tyrosine phosphorylation.

Considering that Ca^{2+} and CaM are involved in the regulation of the acrosomal loss in response to physiological and pharmacological stimulus (Bendahmane et al., 2001; Lopez-Gonzalez et al., 2001; Marin-Briggiler et al., 1999; Si and Olds-Clarke, 2000), participation of CaM kinases in this event was investigated. Motile sperm were suspended in medium not supplemented with Ca^{2+} in the absence or in the presence of KN62 or KN93 (60 μM). After 30 minutes, all the aliquots except the $-Ca^{2+}$ ones, were supplemented with $CaCl_2$ to a final concentration of 2.5 mM. The sperm were incubated in these conditions for 18 hours and then each sample was exposed to 10% human follicular fluid (hFF) or buffer for 1 hour. Sperm incubated in $-Ca^{2+}$ medium were not able to undergo the AR in response to hFF (Fig. 5). In contrast, incubation with CaM kinase inhibitors from the beginning of

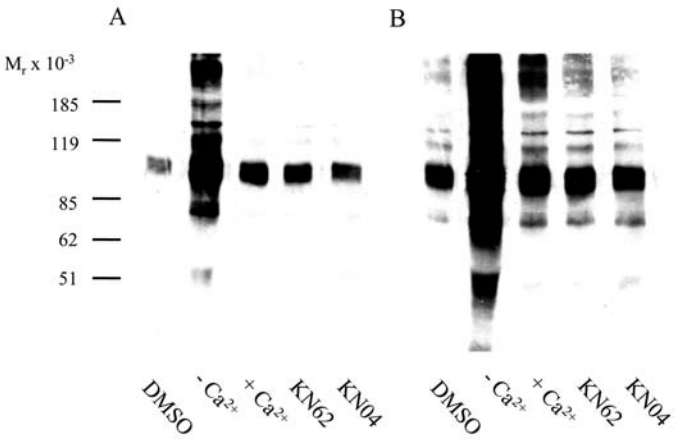

Fig. 4. Effect of KN62 on the Ca^{2+}-associated decrease in protein tyrosine phosphorylation. Immediately after selection using Percoll™ gradients, motile sperm were resuspended in medium not supplemented with $CaCl_2$, and 60 μM KN62 or KN04 were added. After 30 minutes, aliquots were supplemented with $CaCl_2$ (final concentration: 2.5 mM) and cells were incubated for 18 hours under capacitating conditions. Aliquots incubated in medium not supplemented with $CaCl_2$ ($-Ca^{2+}$) and in the absence of the inhibitors ($+Ca^{2+}$) or in the presence of 0.3% DMSO (DMSO) served as controls. Sperm protein extracts (2×10^6 cells per lane) were analysed by PAGE, immunoblotted and probed using a monoclonal antibody against phosphotyrosine (clone 4G10, Upstate Biotechnology). Molecular weight standards ($\times10^{-3}$) are indicated on the left. Panel A, 2-minute exposure; Panel B, 10-minute exposure. A typical experiment is shown. This experiment was performed three times obtaining similar results.

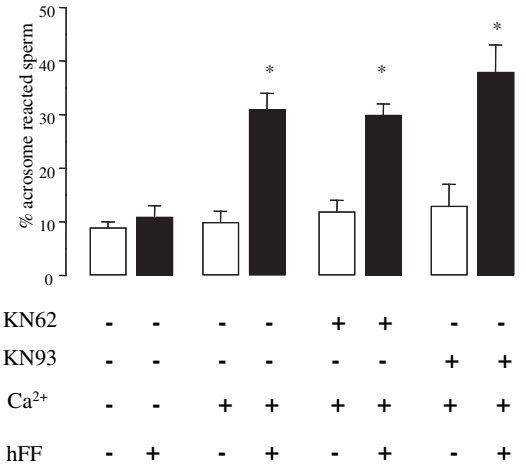

Fig. 5. Effect of CaM kinase inhibitors upon hFF-induced acrosome reaction. After selection using glass wool columns, motile sperm were resuspended in medium not supplemented with $CaCl_2$ in the presence or absence of 60 μM KN62 or KN93. 30 minutes later, aliquots were supplemented with $CaCl_2$ (final concentration: 2.5 mM). Aliquots incubated in media not supplemented with Ca^{2+} ($-Ca^{2+}$) and in the absence of the inhibitors ($+Ca^{2+}$) served as controls. After an 18-hour incubation period, the sperm were exposed to 10% human follicular fluid (hFF) or buffer for 45 minutes and the acrosomal status determined using FITC-*Pisum sativum* agglutinin. The results are expressed as mean±s.e.m., n=4. *P<0.001 compared to acrosomal status of cells with no hFF added under all conditions and cells with hFF but no Ca^{2+}.

Table 2. ATP content of sperm incubated with the CaM kinase inhibitor KN62 or its inactive analogue

Condition	nmoles of ATP per 10^6 sperm	% relative to $+Ca^{2+}$ condition
$-Ca^{2+}$	0.09±0.02*	50
KN62	0.12±0.02*	67
KN04	0.18±0.02	100
DMSO	0.17±0.02	94
$+Ca^{2+}$	0.18±0.02	100

Immediately after selection using glass wool columns, motile sperm were resuspended in medium not supplemented with $CaCl_2$ in the presence or absence of 60 μM KN62 or KN04. After 30 minutes, aliquots were supplemented with $CaCl_2$ (final concentration: 2.5 mM) and cells were incubated for 18 hours in capacitating conditions. Aliquots incubated in medium not supplemented with $CaCl_2$ ($-Ca^{2+}$), in the absence of the inhibitor or the inactive analogue ($+Ca^{2+}$) or with 0.3% DMSO served as controls. ATP content was determined in each aliquot. The results are expressed as mean±s.e.m., n=6.

*P<0.01 when compared to levels in KN04, DMSO and $+Ca^{2+}$ treated cells.

the 18-hour incubation period did not prevent the occurrence of hFF-induced acrosome reaction.

Inhibition of CaM kinases reduces sperm ATP content

One parameter that could affect the maintenance of sperm motility is the availability of ATP. When human sperm were incubated for 18 hours in medium not supplemented with Ca^{2+}, the ATP concentration was significantly reduced compared to cells incubated in the presence of Ca^{2+} (Table 2). Similarly, KN62 but not KN04, reduced the sperm ATP levels when present in the incubation medium (Table 2). These results suggest that CaM kinase antagonists inhibit sperm motility by reducing ATP availability by either a decrease in energy production or by an increased consumption of ATP in other pathways.

Presence, localization and activity of CaM kinase IV in human sperm

The inhibitory effect observed with KN62 and KN93 suggests that CaM kinases are involved in the regulation of human sperm motility. Expression of CaMKIV has been reported in rodent male germ cells (Wu and Means, 2000), though its presence, localization and role in sperm are still unknown. Anti-CaMKIV antibodies were used to determine whether this enzyme is present in human sperm by western blotting. In one-dimensional gels, a protein of 61 kDa was detected (Fig. 6A); two-dimensional western blots detected a series of spots of the expected molecular mass and isoelectric point (Fig. 6B). These data indicate that CaMKIV is present in human sperm. Contrasting with these findings, no protein bands were detected when anti CaMKII was used (data not shown).

To analyse the subcellular localization of CaMKIV in human sperm, indirect immunofluorescence using the same antibody was performed. Sperm incubated with anti-CaMKIV showed a positive signal throughout the flagellum, with a brighter fluorescence in the middle piece (Fig. 6C). Staining was specific, as no signal was evident when normal mouse serum was used (data not shown).

To further investigate the presence of CaMKIV in human sperm, its enzymatic activity was determined using peptide gamma as a substrate. This peptide contains a specific consensus sequence for phosphorylation by CaMKIV (Gringhuis et al., 1997). Sperm incubated for 18 hours in capacitating conditions (Fig. 7, T18) showed a significant increase in the incorporation of ^{32}P to peptide gamma when compared with cells that were resuspended in complete medium and immediately processed for the kinase measurement (Fig. 7, T0). This increase was abolished in sperm incubated in the presence of KN62 during the capacitation period [Fig. 7, T18 (KN62)].

Discussion

The initiation and maintenance of sperm motility is a complex phenomenon that involves the activation of several signal transduction pathways. Evidence from the present study has confirmed that Ca^{2+} ions

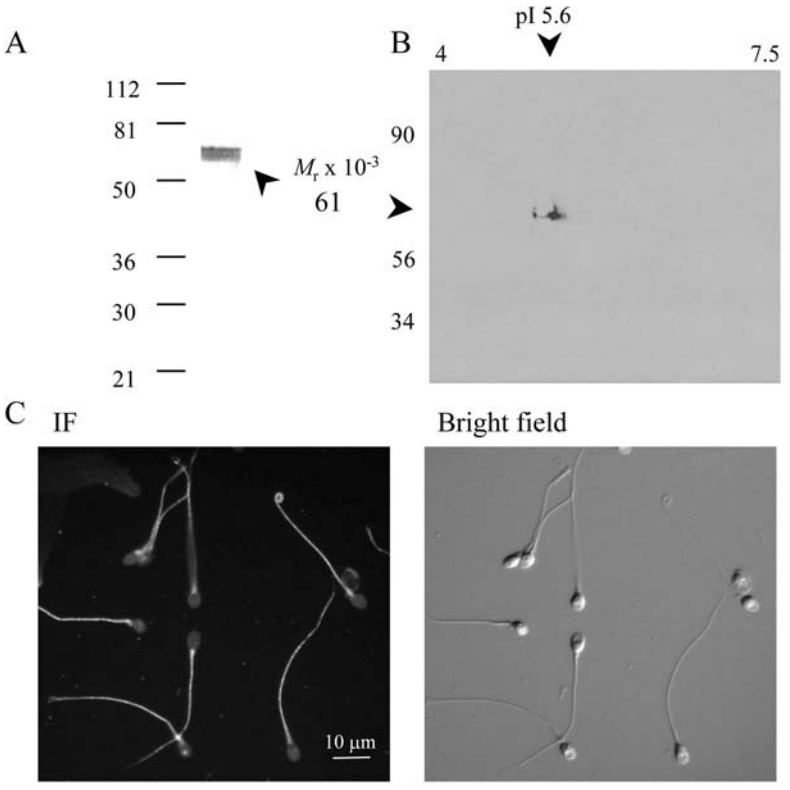

Fig. 6. Presence and localization of CaM kinase IV in human sperm. Human sperm extracts were subjected to one-dimensional (A) and two-dimensional (B) gel electrophoresis, followed by western immunoblotting using a monoclonal anti-CaMKIV antibody (Transduction Laboratories). Both, pI and relative molecular mass ($\times 10^{-3}$) are indicated. This experiment was performed three times obtaining similar results. A typical experiment is shown. (C) Localization of CaMKIV in human sperm by indirect immunofluorescence. Non-capacitated sperm were fixed and processed for indirect immunofluorescence using monoclonal anti-CaMKIV antibody (IF). The corresponding bright field photomicrograph is also shown. Bar, 10 μm.

are required to support human sperm motility, and indicate that members of the CaM kinase family, particularly CaMKIV, are important signalling components in this process. The need for Ca^{2+} in the regulation of sperm function has been widely reported (reviewed by Fraser, 1995). Our results show that when human sperm are incubated in medium not supplemented with $CaCl_2$, progressive motility is significantly reduced, and that 0.22 mM of this cation is sufficient to maintain flagellar motion. Similar Ca^{2+} concentrations are required for the occurrence of some capacitation-related events such as the development of hyperactivated motility (Marin-Briggiler et al., 2003).

Contrasting with the large amount of evidence showing the need of Ca^{2+} for the modulation of mammalian sperm motility, the underlying molecular mechanisms and target proteins have not yet been characterized. CaM has been identified as a component of ciliary and flagellar axonemes, and it has been proposed as the Ca^{2+} sensor that mediates motility in cells from a wide variety of species, including sperm from mammals and echinoderms (Brokaw, 1991; Lindemann et al., 1991; Plattner and Klauke, 2001; Si and Olds-Clarke, 2000). In humans, CaM concentration has been shown to be decreased in asthenozoospermic patients (Reyes et al., 1987). Additionally, some CaM inhibitors negatively affected sperm motility parameters and led to a reduction in the percentage of motile and hyperactivated cells (Ahmad et al., 1995; Aitken et al., 1988). Recently, six different CaM antagonists were shown to inhibit the agonist-induced acrosome reaction in mouse sperm (Zeng and Tulsiani, 2003); three of these compounds (48/80, W13 and the CaM-binding domain) had no effect on sperm motility or tyrosine phosphorylation. In contrast, three others (W7, ophiobolin A and calmidazolium) inhibited tyrosine phosphorylation and sperm motility without altering viability. These results suggest that CaM is involved in the regulation of different sperm parameters. Because this protein can interact with several enzymes, it is predicted that CaM antagonists could prevent the activation of multiple pathways.

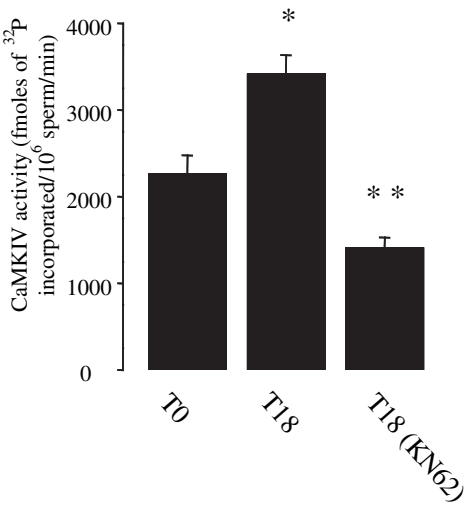

Fig. 7. CaM kinase IV activity in human sperm. CaMKIV activity was measured in sperm treated immediately after resuspension in the Ca^{2+}-containing medium (T0) or in sperm incubated in the same medium for 18 hours in the absence (T18) or presence of 60 μM KN62 [T18 (KN62)]. Sperm subjected to these different experimental conditions were washed twice with PBS, cell concentration was adjusted to 10^7 cells/ml, and aliquots were stored at –20°C until use. Enzyme activity was measured in these extracts using peptide gamma and [γ-^{32}P]ATP as substrates. The results are expressed as mean±s.e.m., n=7. *P<0.001 compared to CaMKIV activity in T0 and T18 (KN62) cells; **P<0.01 compared to CaMKIV activity in T0 and T18 cells.

Among the multiple targets of CaM are the family of CaM kinases. A recent study in *Chlamydomonas* has shown that Ca^{2+} controls dynein-driven microtubule sliding through the activation of axonemal CaM and CaM kinase, as dynein activity was abolished when isolated axonemes were exposed to CaM or CaMKII inhibitors (Smith, 2002). In the present report, using membrane-permeable inhibitors and their inactive analogues as controls, we were able to demonstrate the involvement of CaM kinases in the regulation of human sperm motility. In the presence of the active inhibitor KN62, a significant and specific decline in progressive motility and a reduction in sperm motion parameters were observed. When sperm were incubated with KN93 CaM kinase inhibitor, motility was also negatively affected, although the effect was not as pronounced as that observed with KN62. Such results could not be attributed to differences in inhibitors affinity, as similar *Ki* values have been reported for both inhibitors to all CaM kinases studied (Hidaka and Yokokura, 1996). However, the idea that KN93 is unable to enter the sperm tail efficiently cannot be discarded.

By immunological and biochemical studies, we were able to detect the presence of active CaMKIV in the flagellum of human sperm. In previous studies, CaMKIV was found in mouse spermatids, where it is localized to the nucleus, and participates in chromatin remodelling (Wu and Means, 2000). Interestingly, changes in the subcellular localization of CaMKIV (translocation from the nucleus into the cytoplasm) have been described when granulosa cells differentiate into luteinized cells (Wu et al., 2000b). Although there is no direct evidence of CaMKIV translocation in the male gamete, the existence of similar mechanisms during cellular differentiation could explain the localization of CaMKIV in the flagellum of mature sperm. Localization of CaMKIV in the sperm tail is consistent with the role proposed for this enzyme in human sperm motility regulation. In addition to CaMKIV, immunodetection of CaMKII was attempted in whole cells and in protein sperm extracts. Although efforts to find this enzyme in human sperm were not successful, the presence of CaMKII as well as CaMKI in these cells cannot be completely discarded.

Mammalian sperm motility is regulated by the phosphorylated status of flagellar proteins (Carrera et al., 1996; Si, 1999; Tash and Bracho, 1994; Vijayaraghavan et al., 1997). Several lines of evidence have shown that Ca^{2+} participates in the regulation of protein phosphorylation on tyrosine residues (Visconti and Kopf, 1998). In human cells, it has been proposed that Ca^{2+} reduces the level of protein tyrosine phosphorylation through the activation of calcineurin, a CaM-dependent serine/threonine phosphatase (Carrera et al., 1996) or by decreasing intracellular ATP availability (Baker et al., 2004). Moreover, participation of CaM in the signalling events leading to protein tyrosine phosphorylation has been described in mouse gametes (Zeng and Tulsiani, 2003). The effect of CaM antagonists on tyrosine phosphorylation are probably related to other CaM target enzymes, which are different to CaM kinases,

because either KN62 or KN93 do not modify tyrosine phosphorylation pattern of human sperm. The findings from these studies also suggest that CaM kinases are involved in sperm motility by modulating intracellular pathways not related with the regulation of tyrosine phosphorylation.

In addition to Ca^{2+} signalling, the cAMP/PKA pathway plays a key role in the regulation of mammalian sperm motility and protein tyrosine phosphorylation (Luconi and Baldi, 2003; Turner, 2003). In the present study, the relationship between CaM kinases and cAMP-activated events was analysed by incubating human sperm with a CaM kinase inhibitor in the presence of agonists of cAMP/PKA pathway. Such compounds were unable to overcome KN62-mediated inhibition of sperm motility. These results agree with previous findings in the mouse that show that exogenous cAMP analogues could not reverse motility inhibition caused by CaM antagonists (Si and Olds-Clarke, 2000), suggesting that CaM and CaM kinase regulation of flagellar movement is independent or downstream of cAMP. Consistent with the independence of these two pathways in the regulation of mammalian sperm motility, it was recently demonstrated in bull gametes that although induction of hyperactivation depends on the presence of Ca^{2+}, it does not require the activation of the cAMP/PKA pathway (Marquez and Suarez, 2004). Similarly, in human sperm, Ca^{2+} is required for the development of hyperactivated motility (Marin-Briggiler et al., 2003). However, in this study, we did not find specific effects of CaM kinase inhibitors on hyperactivated parameters. Although there is a reduction in hyperactivation when human sperm are incubated in the presence of KN62, this reduction is also observed in the presence of KN04, the inactive KN62 analogue, suggesting that this effect is not related to the inactivation of CaM kinases.

This work provides evidence suggesting that members of the CaM kinase family are involved in the Ca^{2+} regulation of human sperm motility; however, the mechanism by which these kinases are involved in this regulation is not clear. One possibility is that the maintenance of energy levels is dependent on CaM kinase regulation. In this respect, human sperm incubated in media not supplemented with Ca^{2+} ions showed a significant reduction in ATP levels. This decrease was also observed in the presence of KN62 but not in the presence of KN04 suggesting the involvement of CaM kinases in the regulation of ATP metabolism. At what level this is controlled is not known, and it is possible that CaM kinases are involved in the regulation of the sperm glycolytic pathway. Further studies are needed to understand the molecular mechanisms by which CaM kinases regulate sperm motility and to clarify the relationship between this and other previously reported pathways.

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