Transient receptor potential (TRPC) channels in human sperm: expression, cellular localization and involvement in the regulation of flagellar motility

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Abstract Capacitative Ca^{2+} entry is a process whereby the activation of Ca^{2+} influx through the plasma membrane is triggered by depletion of intracellular Ca^{2+} stores. Some transient receptor potential (TRPC) proteins have been proposed as candidates for capacitative Ca^{2+} channels. Recent evidence indicates that capacitative Ca^{2+} entry participates in the sperm acrosome reaction (AR), an exocytotic process necessary for fertilization. In addition, several TRPCs have been detected heterogeneously distributed in mouse sperm, suggesting that they may participate in other functions such as motility. Using reverse transcription-polymerase chain reaction (RT-PCR) analysis, RNA messengers for TRPC1, 3, 6 and 7 were found in human spermatogenic cells. Confocal indirect immunofluorescence revealed the presence of TRPC1, 3, 4 and 6 differentially localized in the human sperm, and immunogold transmission electron microscopy indicated that TRPC epitopes are mostly associated to the surface of the cells. Because all of them were detected in the flagellum, TRPC channel antagonists were tested in sperm motility using a computer-assisted assay. Our results provide what is to our knowledge the first evidence that these channels may influence human sperm motility.

Key words: Ca^{2+} channel; Capacitative Ca^{2+} entry; Transient receptor potential; TRPC; Store operated channel; Sperm motility

1. Introduction

Capacitative Ca^{2+} entry in some mammalian cell types helps to maintain Ca^{2+} levels in intracellular stores that may otherwise be depleted during production of repetitive intracellular Ca^{2+} (Ca^{2+}i) spikes. However, ion influx through this pathway can also generate Ca^{2+} signals important for other functions [1]. Recent evidence suggests that the protein encoded by the transient receptor potential (*trp*) gene expressed in *Drosophila* photoreceptors may be homologous with capacitative Ca^{2+} entry channels in mammals, usually known as store operated channels (SOCs). In mammals, the TRP channel superfamily is constantly growing and encompasses three main families according to structural motifs (TRPC, TRPV and TRPM) [1,2].

TRPC channels are widely expressed in the nervous system as well as in non-excitable cells and play fundamental roles in processes ranging from sensory physiology to male fertility. In mouse spermatogenic cells (SC), several kinds of mRNAs for TRPCs (*TRPC1–7*) have been reported [3]. As for the proteins, immunocytochemical studies suggest the presence of TRPC1, 3 and 6 in the mature mouse sperm [3]. In addition, the presence of TRPC2 has been also detected in mouse sperm and shown to functionally mediate Ca^{2+} influx during the acrosome reaction (AR) [4]. In mouse, the AR is triggered by ZP3, a glycoprotein of the egg's *zona pellucida* (ZP), causing a transient Ca^{2+} influx into sperm through voltage-gated T-type Ca^{2+} channels. This initial response promotes a sustained increase in Ca^{2+}i that drives the AR [5]. TRPC2 has been proposed to participate in the sustained sperm Ca^{2+} influx triggered by ZP3 [4]. Other TRPCs or unknown subunits may substitute for TRPC2 since *TRPC2*$^{-/-}$ mutant mice are fertile [6]. It is worth mentioning that the human *TRPC2* gene appears to be a pseudogene, considering that several expressed sequence-tagged clones (ESTs) show early stop codons [7].

Interestingly, it has been found that TRPC channels are not only localized to the sperm head, but several of these proteins are present in the flagellum, suggesting that they may serve sperm to regulate important Ca^{2+}-dependent events in addition to the AR. Although the function of the different TRPC channels in the sperm flagellum is unknown, it has been speculated that they could participate in significant events for fertilization such as the initiation of motility (activation) and hyperactivation [3]. It is well known that Ca^{2+}, acting directly on the flagellum axoneme, is a key regulator of sperm motility and hyperactivation [8], however the mechanism by which Ca^{2+} reaches the axoneme to modulate motility has not been identified. In this report, we show that TRPC Ca^{2+}-permeable channels are expressed and differentially localized to the mature human sperm head and importantly to the flagellum, therefore they may influence flagellar movement.

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This is consistent with alterations in the swimming characteristics of sperm induced by TRPC channel antagonists.

2. Materials and methods

2.1. Ejaculates

Human sperm were obtained from normal fertile volunteers who had authorized the use of their semen following an appropriate routine of donation. The experimental design complied with the precepts of the Helsinki Declaration (1975).

2.2. Ribonucleic acid (RNA) extraction and polymerase chain reaction (PCR)

Total RNA was isolated from the ejaculate of normozoospermic volunteers using Trizol reagent (Gibco BRL) and cDNA was synthesized using the Superscript first strand synthesis system (Invitrogen) for reverse transcription (RT)-PCR according to the manufacturer's instructions. Specific primers for TRPC channels were designed based on coding sequences, and the predicted sizes of the fragments are shown in Table 1. The typical PCR temperature profile was 40 cycles of amplification (94°C for 1 min, 55°C for 1 min, and 72°C for 30 s), and a final extension at 72°C for 3 min. The PCR products were separated on agarose gels, purified and sequenced. Sequence identities were established by searching the databases using the NCBI BLAST programs.

2.3. Indirect immunofluorescence (IF)

Sperm immunolocalization of TRPC channels was assessed by fluorescence microscopy as previously described [3]. In brief, human sperm were fixed in 5% formaldehyde in phosphate-buffered saline (PBS) and then attached to Teflon printed glass slides coated with a bioadhesive. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and incubated for at least 30 min in a blocking solution. Samples were then incubated overnight at room temperature with primary antibodies at a 1:200 dilution (or 1:50 for TRPC6). Samples were next incubated for 1 h at room temperature with Alexa594 or Alexa488 (Molecular Probes Inc.) conjugated secondary antibody. Sperm were examined by confocal fluorescence and phase contrast microscopy.

2.4. Sample preparation and transmission electron microscopy (EM)

Sperm were fixed in a 4% paraformaldehyde–0.5% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.3) for 2 h and stored at 4°C until processed. Cells were dehydrated in 50% ethanol for 20 min and then in 60–100% ethanol. The final pellet was embedded in LR white resin (Electron Microscopy Sciences) 5 days at 50°C. Ultrathin sections were cut and mounted onto nickel grids. Sections were first incubated in a blocking solution containing 5% fetal bovine serum and 0.005% Tween 20, and then incubated overnight with TRPC channel-specific primary antibodies (Alomone Labs) diluted 1:20 in PBS. Subsequently, samples were incubated 1 h with a goat anti-rabbit IgG secondary antibody (1:40) conjugated to 20 nm colloidal gold particles (ZYMED). The sections were counterstained with uranyl acetate and lead citrate and then examined using a Phillips EM-201 electron microscope.

2.5. Swim-up preparation and evaluation of sperm motility

After liquefaction, 1 ml of Ham's F-10 was applied to 1 ml of semen, after 1 h incubation at 37°C, the upper layer of the medium was separated. The concentration of the cell suspension was adjusted to $8–12\times10^6$ cells/ml. Video-recording of 15–20 microscopic fields of the sperm sample was made over 1 min using phase contrast microscopy. For each sample, ~1200 motile sperm were tracked and analyzed with the Hamilton–Thorne HTM-IVOS-12 computer-assisted semen analysis (CASA) system. Thirty frames were acquired at a frame rate of 60 Hz. Sperm motility parameters measured included progressive velocity (VSL; the straight-line distance from the beginning to the end of each sperm's track divided by the time elapsed, expressed in μm/s), the path velocity (VAP; the total distance along the smoothed average path for each sperm divided by the time elapsed, in μm/s), the curvilinear velocity (VCL; the track speed, or total distance covered by each sperm divided by the time elapsed, in μm/s), amplitude of lateral head displacement (ALH, in μm), beat cross frequency (BCF, in Hz) and the derived parameters of linearity (LIN = VSL/VCL × 100%) and straightness (STR = VAP/VCL × 100%). TRPC blockers were prepared as 100 mM stock solutions in distilled water (Ni^{2+}, Gd^{3+} and SKF96365) or dimethyl sulfoxide (DMSO) (2-aminoethoxydiphenyl borate; 2-APB).

3. Results

AR in mammals is triggered during gamete contact and causes a transient Ca^{2+} entry into sperm through voltage-gated (Ca_V) channels which is followed by a second Ca^{2+} influx that promotes a sustained increase in Ca^{2+}i. Mammalian TRPCs have emerged as candidates for the ion channels that mediate this sustained Ca^{2+} influx, however their expression and cell distribution have not been established in human sperm [9]. Therefore, it seemed necessary to determine which members of the TRPC channel family are present in these cells.

Sperm are small differentiated terminal cells unable to synthesize proteins; all their ion channels are made during spermatogenesis. SC are being used to study sperm ion channels since they are larger and molecular biology techniques can be applied [9]. Obtaining human SC is difficult [10], however, semen from fertile men contains ~5% non-sperm cells (called round cells) [11] $>20\times10^6$ cells/ml, there are at least 1×10^6/ml round cells. More than 94% of the round cells in human ejaculates are germinal and those remaining are epithelial and blood cells [12]. Thus, the mRNAs found in ejaculates are derived mainly from the SC since mature sperm have only a few mRNAs [13]. Here, a set of oligonucleotides (Table 1) was designed to amplify the six different known mammalian *TRPC* genes from human ejaculates (*hTRPC2* is a pseudogene) using RT-PCR. Fig. 1 shows a 1% agarose gel with ethidium bromide staining of the PCR products for *TRPC1*, *3*, *6*, and *7* from human ejaculate cDNA. The PCR products were sequenced, and the identities of the amplicons were verified by database homology searches. These data indicate that multiple *TRPC* genes are expressed in human SC.

We used antibodies against TRPC1, 3, 4 and 6, whose specificity has been tested previously [3,9], to investigate their expression at the protein level in human sperm. Fig. 2A shows a confocal IF image of human sperm treated with antibodies

Table 1
Sets of primers designed to amplify *TRPC* homologues from human SC

Gene	Forward primer 5′–3′	Reverse primer 5′–3′	T_m (°C)/size (bp)
TRPC1	GCTAATGCCACGAAGT	CGTTTGGGTGAGGACA	55/471
TRPC3	GACATATTCAAGTTCATGGTCCTC	ACATCACTGTCATCCTCAATTTC	55/323
TRPC4	CTGCAAATATCTCTGGGAAGA	GCTTTGTTCGTGCAAATTTCC	53/411
TRPC5	GCTCGCAGCCACCCCAAAGGGAGGA	CCAATGTCCCTACCCTGTTCTCCCAGCTCTC	65/523
TRPC6	GACATCTTCAAGTTCATGGTCATA	GACATCTTCAAGTTCATGGTCATA	53/320
TRPC7	TGCTGCTCAAGGGTGC	CTGCTGACAGTTAGGGT	52/443

The predicted sizes and melting temperatures of the PCR products (T_m) are indicated on the right.

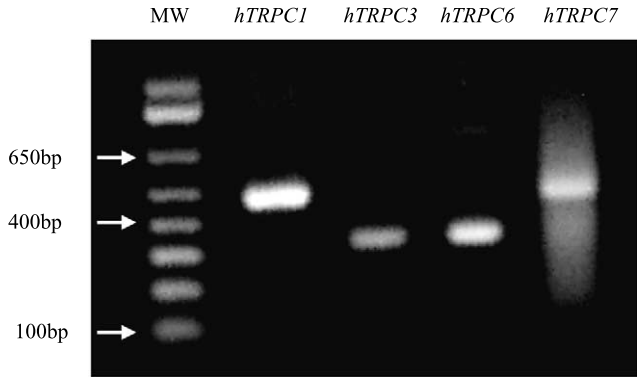

Fig. 1. RT-PCR analysis of TRPC mRNAs expression in human semen. MW, molecular weight markers; the order of the lanes is: PCR products for *hTRPC1*, *3*, *6* and *7*.

directed to the TRPC1 protein. As can be seen the principal piece of the flagellum is heavily stained, showing uniform fluorescence, although no fluorescent signal was detected in the sperm midpiece. Additionally, in some cells TRPC1 protein showed a ring-shaped pattern at the posterior acrosomal region in the sperm head along the equatorial segment (not shown). Both TRPC3 (Fig. 2B) and TRPC4 (Fig. 2C) gave a strong signal in the midpiece. TRPC3 heavily stained the acrosome region, sometimes with a punctuate aspect, and TRPC4 showed a weak diffuse signal in the head and the principal piece. Sperm incubated with the anti-TRPC6 showed strong staining at the midpiece, punctuate pattern in the principal piece and a weak and diffuse signal in the head (Fig. 2D). The specificity of the TRPC antisera was demonstrated by the absence of signal in sperm incubated with antisera (1:2 for TRPC1, 3 and 4; 1:6 for TRPC6) that had been preincubated with their corresponding peptide antigens (not shown).

To ensure expression and confirm TRPC protein localization, EM was next performed on the human sperm. Ejaculated sperm were embedded and ultrathin sections were incubated with TRPC antisera followed by a secondary antibody conjugated to colloidal gold. The micrographs shown in Fig. 3 support our observations at the confocal microscopic level and illustrate that TRPC1, 3, and 4 are localized in both the heads and flagella with no specific labeling detected in the sperm nucleus. Furthermore, gold particles did not appear to be distributed in the cell interior, instead TRPC1, 3, and 4 seemed to be preferentially associated with the plasma membrane in the different regions of the cell. Control sections incubated with antibodies blocked by the peptide antigen showed only background levels of gold particles with no specific localization in the sperm. However, while the TRPC6 signal was abolished by competition with its corresponding peptide antigen in IF experiments, it was not competed in EM (not shown).

Immunoconfocal and EM images showed that TRPC channels in human sperm are strongly expressed in the flagellum, suggesting that they may serve to regulate important Ca^{2+}-dependent events in this region such as flagellar beating. Therefore, we next used a CASA system to assess the participation of TRPC channels on sperm motility using pharmacological agents that inhibit their function.

A swim-up sperm suspension was diluted in Ham's F-10 medium alone (control) or containing different concentrations of Ni^{2+} or SKF96365 and motility analyses were performed. Ni^{2+} has been shown to block the mouse sperm SOCs involved in the AR with a $K_i \sim 500$ μM [5]. After 1 min incubation, 1 mM Ni^{2+} caused a ~20% reduction in the number of swimming cells ($K_i \sim 218$ μM) (Fig. 4A). In addition, this treatment decreased the following parameters in the remaining motile sperm (Fig. 4B): fast motility, VSL and LIN. Similarly, SKF96365, a blocker of TRPC channels, significantly decreased sperm motility in a dose- and time-dependent manner. Application of 20 μM SKF96365 decreased the percentage of motile sperm by ~30% after 1 min incubation ($P < 0.05$) and differentially affected the individual motility parameters (Fig. 4C and D). This SKF96365 concentration inhibited 68.9 ± 9%

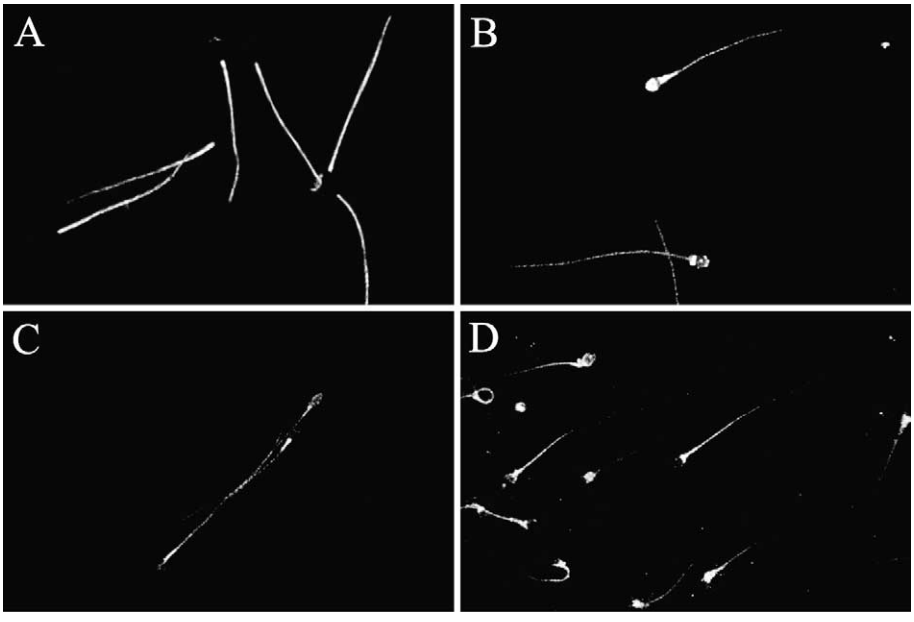

Fig. 2. Identification and immunolocalization of TRPC channels in human sperm. Confocal fluorescence images of sperm labeled with specific anti-TRPC1 (A), anti-TRPC3 (B), anti-TRPC4 (C) and anti-TRPC6 (D) antibodies illustrating the staining pattern of the proteins.

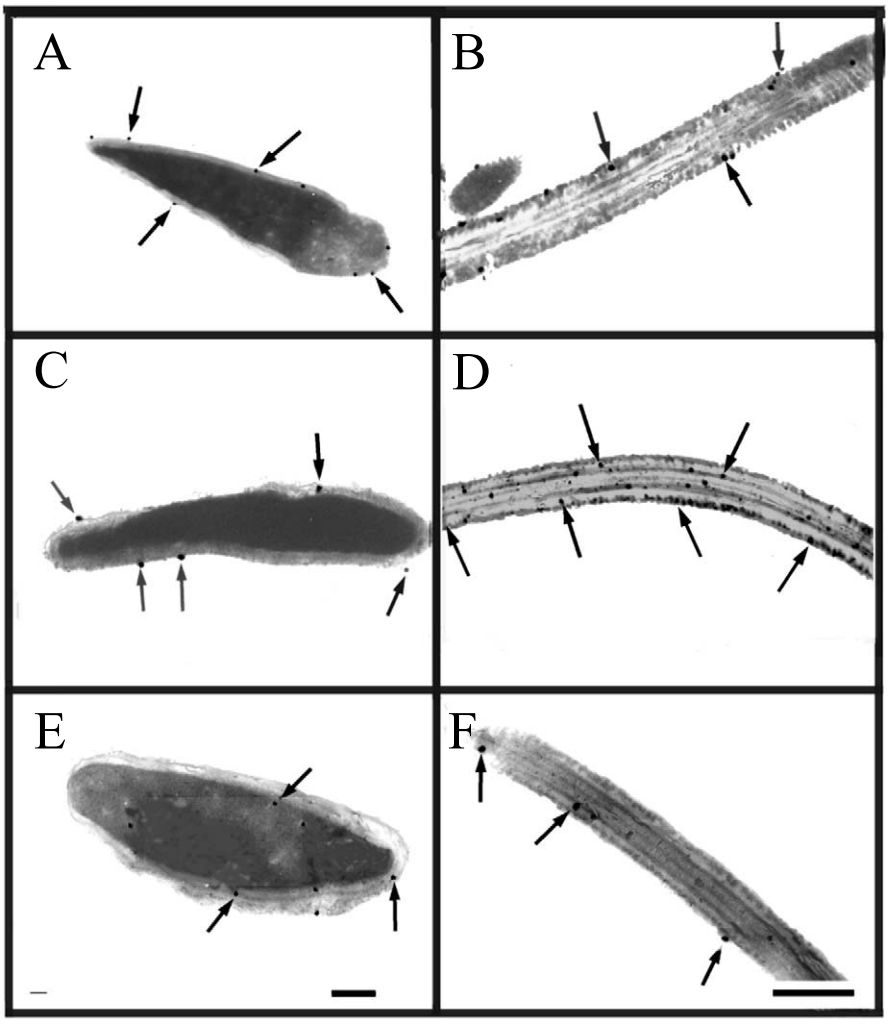

Fig. 3. Immunogold EM of TRPC channels in human sperm. Longitudinal sections showing the subcellular localization of the TRPC1 (A, B), TRPC3 (C, D) and TRPC3 (E, F) antigens in the head and principal piece of the flagellum, respectively. The arrows indicate the gold particles that label principally the periphery of the cells, though TRPC3 antibody also labeled dense fibers and the ribs of the fibrous sheath in the flagellum. Scale bars = 0.4 μm.

of the motility after a 10 min incubation (Fig. 4C). In contrast, 20 μM SKF96365 did not affect the magnitude of the voltage-gated T-type current expressed in mouse SC (3 min incubation, not shown). Lastly, we also tested the TRPC channel blockers $GdCl_3$ and 2-APB in the 0.5–5 and 10–75 μM range, respectively, but they proved ineffective in altering the sperm motility parameters (not shown).

4. Discussion

Diverse studies in mammalian sperm indicate that an increase in $Ca^{2+}i$ is critical for several physiological processes during fertilization, such as sperm motility, capacitation and AR [9]. However, little is known about the molecular identity of the pathways that regulate these events. Recently, the presence of multiple types of TRPC mammalian homologues in mouse SC and sperm was revealed [3]. A recent report indicates that SOCs regulate chemotactic behavior in ascidian sperm [14]. However, in human sperm TRPC channels have not been identified and their possible functions are yet to be defined. The findings presented here provide what is to our knowledge the first evidence for the expression of different TRPC channels in human sperm and for their possible involvement in the motility in these cells.

Positive immunolocalization of the TRPC proteins occurred importantly in the tail region of human sperm, though in some cases the heads were also immunopositive. These data provided evidence of heterogeneity in the expression of TRPC channels in human sperm and suggested that they may participate in $Ca^{2+}i$ homeostasis and play several physiological roles in these cells. Our immunocytochemistry results are basically consistent with the PCR data since we were able to detect TRPC1, 3 and 6 both at the RNA and protein level. The inconsistency between the IF and EM experiments regarding TRPC6 may be due to technical problems associated with the use of a different secondary antibody. The presence of TRPC6 should be confirmed by Western blot. Although we also identified messenger for *TRPC7*, we could not confirm the presence of this channel in sperm due to the lack of specific antibodies. Likewise, we detected TRPC4 with antibodies but we were unable to amplify the corresponding mRNA, even though the oligonucleotides used amplified *TRPC4* from mouse brain and HEK293 cells (not shown). Thus, the reason for this discrepancy is unknown; perhaps it lies within

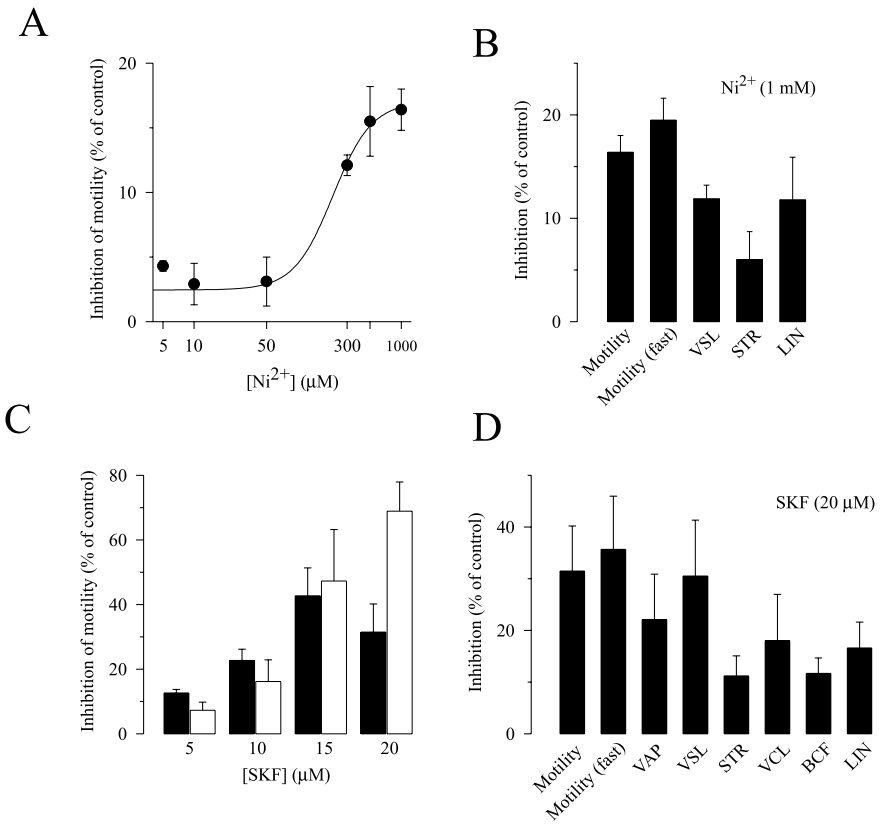

Fig. 4. CASA parameters of individual human sperm from ejaculates in the presence of TRPC inhibitors. A: Concentration-dependent inhibition of sperm motility by Ni^{2+}. The inhibition curve was fitted with the Hill equation. B: The effects of the Ni^{2+} treatment on the velocity parameters of sperm classified as average and fast motility as well as VSL, STR and LIN are shown ($n = 10$). C: Concentration- and time-dependent inhibition of sperm motility by SKF96365. Data represent the percentage of inhibition (mean ± S.E.M.) after 1 (filled bars, $n = 7$) and 10 min (open bars, $n = 3$) of SKF96365 treatment. D: The CASA parameters of sperm classified as: average motility, fast motility, VAP, VSL, STR, VCL, BCF and LIN obtained in the presence of drug are shown. The effects of both antagonists were evaluated as the ratio between the posttreatment value and the control swim-up value ($n = 7$).

the regulation of the mRNA decay [15]. Alternatively, there may be a sperm-specific TRPC4 isoform. Since we could not detect *TRPC5* messenger and lacked specific antibodies for this channel, its presence needs to be explored further.

Previous studies have shown reactivity of TRPC antibodies not only in the head but also in the flagellum of mouse sperm [3] suggesting that they may be implicated in determining sperm motility. Since Ni^{2+} blocks the SOCs involved in the mouse AR (IC_{50} of ~500 μM) [5], we tested its capacity to inhibit human sperm motility. We found that though mildly, Ni^{2+} affected cell motility in a dose-dependent fashion, decreasing it by ~20% at 1 mM and differentially altering the motility parameters of swimming sperm. Ni^{2+} at this concentration could be affecting basal Ca^{2+} through SOCs partially activated in swimming sperm. This concentration of Ni^{2+} could also influence Ca^{2+}i altering other Ca^{2+} transport systems such as the Na^+/Ca^{2+} exchanger [16] and the other TRPCs present in the flagella. On the other hand, it seems that T-type Ca^{2+} channels which are more sensitive to Ni^{2+} [17], do not strongly affect basal sperm motility. The participation of other transporters that elevate Ca^{2+}i such as non-T-type channels as well as channels from intracellular stores [18] or plasma membrane non-selective cation channels regulated by cAMP [19,20] cannot be ruled out.

To further establish the pharmacological profile of human sperm ion channels involved in motility, another TRPC blocker, SKF96365, was tested. This compound reduced motility in a concentration- and time-dependent fashion. Remarkably, a 10 min incubation with 20 μM SKF96365 inhibited ~70% of sperm motility. The estimated IC_{50} of ~7 μM for this inhibition is consistent with the reported efficacy of SKF96365 to block recombinant TRPC channels (IC_{50} 5–8 μM) [21,22]. It is worth mentioning that 20 μM SKF96365 did not significantly affect Ca_V T-type channel activity recorded in mouse SC, suggesting that TRPC channels can indeed influence sperm motility.

Our findings with Ni^{2+} and SKF96365 indicate that TRPC channels may influence flagellar motility. In this scenario, the results with two other SOC antagonists, Gd^{3+} and 2-APB, present something of a paradox since they do not appear to affect sperm motility. Possibly this unexpected finding arises from the complex interactions between these two compounds and the TRPCs. Micromolar Gd^{3+} has been reported to increase TRPC4- and TRPC5-mediated currents, while mM Gd^{3+} inhibits them [23]. In addition, recombinant heterologously expressed hTRPC3 is completely insensitive to this lanthanide [24]. Likewise, 2-APB only partially inhibits some TRPC channels at concentrations as high as 100 μM [24]. It has been speculated that these pharmacological features may result from the different modes of activation of the channels and may also reflect differences in their molecular structure. The sperm TRPC channels that modulate motility may be

different from those responsible for capacitative Ca^{2+} entry such as receptor operated, second messenger activated TRPC channels (TRPC3, 6 and 7). Alternatively it remains possible that the sperm TRPCs implicated in motility are heteromultimers with a peculiar pharmacological profile.

Recent evidence indicates that TRPC2 participates in the sustained Ca^{2+} entry during AR in mice [4]. Unexpectedly, genetic ablation of *mTRPC2* did not compromise the fertile capability of null mice [6,25]. These findings suggest that the ion channel involved in the sustained increase in Ca^{2+}i necessary for the AR may be a tetramer composed of different TRPC proteins. Furthermore, in humans *TRPC2* is considered a pseudogene [26], thus TRPC1, 3, 4 and 6 present in the sperm head would be candidates to form the SOC involved in the human sperm AR.

Lastly, though the external signals that modulate sperm motility are not well understood, our observations are consistent with the known fundamental role of Ca^{2+} in motility. The balance of Ca^{2+}i can be influenced by TRPC channels that have a wide range of regulatory modes. It remains for future studies to determine the exact mechanisms whereby TRPCs contribute to Ca^{2+} entry to sperm, and the role these proteins play in the regulation of cellular metabolism and responsiveness to extracellular signals during sperm motility.

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