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Expression and differential cell distribution of low-threshold Ca^{2+} channels in mammalian male germ cells and sperm

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Abstract Numerous sperm functions including the acrosome reaction (AR) are associated with Ca^{2+} influx through voltage-gated Ca^{2+} (Ca_V) channels. Although the electrophysiological characterization of Ca^{2+} currents in mature sperm has proven difficult, functional studies have revealed the presence of low-threshold (Ca_V3) channels in spermatogenic cells. However, the molecular identity of these proteins remains undefined. Here, we identified by reverse transcription polymerase chain reaction the expression of $Ca_V3.3$ mRNA in mouse male germ cells, an isoform not previously described in these cells. Immunoconfocal microscopy revealed the presence of the three Ca_V3 channel isoforms in mouse spermatogenic cells. In mature mouse sperm only $Ca_V3.1$ and $Ca_V3.2$ were detected in the head, suggesting its participation in the AR. $Ca_V3.1$ and $Ca_V3.3$ were found in the principal and the midpiece of the flagella. All Ca_V3 channels are also present in human sperm, but only to a minor extent in the head. These findings were corroborated by immunogold transmission electron microscopy. Tail localization of Ca_V3 channels suggested they may participate in motility, however, mibefradil and gossypol concentrations that inhibit Ca_V3 channels did not significantly affect human sperm motility. Only higher mibefradil doses that can block high-threshold (HVA) Ca_V channels caused small but significant motility alterations. Antibodies to HVA channels detected $Ca_V1.3$ and $Ca_V2.3$ in human sperm flagella.

Key words: Ca^{2+} channel; Acrosome reaction; Sperm motility; Gossypol; Mibefradil

1. Introduction

Sperm motility, capacitation and the acrosome reaction (AR) are fundamental processes for fertilization [1–4]. The effects of certain physiological agonists of these processes are mediated by changes in the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) [1–5]. In particular, the mouse AR is associated with a zona pellucida-induced fast transient $[Ca^{2+}]_i$ increase (~250 ms) involving the activation of low-threshold activated (LVA or T-type) Ca^{2+} channels which is followed by a sustained increase lasting minutes that is linked to store-operated channel (SOC) opening [6]. In spite of the crucial role of LVA channels in sperm physiology [3,7,8], their definitive molecular identification and localization remains elusive. This issue is further complicated by reports indicating that high-threshold activated (HVA) channels are also present and functional in mammalian spermatogenic cells and mature sperm [9].

The inability of sperm to synthesize proteins impedes the use of standard molecular approaches. Their minute size, complex geometry and motile nature have precluded a systematic electrophysiological characterization. For these reasons, more recent efforts have focused on the germ-line cells, the progenitors of sperm [3,7,8]. Three different classes of LVA Ca^{2+} channels (known as $Ca_V3.1$–$Ca_V3.3$) have been cloned and expressed [10–12]. The presence of mRNAs for $Ca_V3.1$ and $Ca_V3.2$, but not for $Ca_V3.3$, has been previously documented in mouse [13,14] and human [15] spermatogenic cells. None of these channels has been immunolocalized in sperm. Very recently, using reverse transcription polymerase chain reaction (RT-PCR) the expression of the mRNAs for all Ca_V3 channels in motile human sperm was detected [16]. Here, we report the detection of a transcript for $Ca_V3.3$ from mouse male germ cells and the distribution of the different low-threshold Ca^{2+} channel proteins in mouse spermatogenic cells and mature mouse and human sperm. Consistent with the expression of different low-threshold Ca^{2+} channel proteins, Ni^{2+} inhibition of whole-cell patch-clamp native currents in mouse spermatogenic cells unveiled the presence of subtypes of Ca_V3 channels in these cells.

Lastly, since $Ca_V3.2$ and $Ca_V3.3$ channels are expressed prominently in sperm flagella, studies were carried out to explore their involvement in motility. Although gossypol had previously been shown to inhibit motility in human sperm

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[17], our results using computer-assisted sperm motility analysis (CASA) showed that concentrations of mibefradil [11] and gossypol [18] that inhibit low-threshold Ca^{2+} channels in mouse spermatogenic cells did not reduce basal motility. Other Ca^{2+}-permeable channels like Catsper and SOCs have been shown to modulate this important function [19–21]. Higher mibefradil concentrations that can block L-type HVA channels inhibited sperm motility and antibodies to HVA channels detected $Ca_V1.2$ and $Ca_V2.3$ in human sperm flagella. However, other blockers of L-type channels such as nifedipine and calciseptine did not significantly affect sperm basal motility.

2. Materials and methods

2.1. RT-PCR

The isolation of RNA from mouse spermatogenic cells, preparation of cDNA and RT-PCR were conducted as described previously [22,23]. The PCR primers employed were: sense 5′-ATCTGCTCCC-TGTCGG-3′ and antisense 5′-GAGAACTGGGTCGCTATG-3′. The conditions employed were as follows: after an initial treatment for 5 min at 94°C, the following cycle was repeated 40 times: 30 s at 94°C, 1 min at 54°C, and 1 min at 72°C. Final extension was 5 min at 72°C. cDNA fragments amplified by PCR were analyzed in a 1% agarose gel; the expected size for the PCR product corresponding to $Ca_V3.3$ was 404 bp.

2.2. Immunolocalization of Ca^{2+} channels in spermatogenic cells and sperm

2.2.1. Controls (not shown). HEK-293 cells stably expressing α_{1G} ($Ca_V3.1$), α_{1H} ($Ca_V3.2$) and α_{1I} ($Ca_V3.3$) channels [24] were used to determine the specificity of the primary antibodies (anti-$Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$ from Santa Cruz Biotechnology, and anti-$Ca_V3.1$ from Alomone Labs). Anti-$Ca_V3.2$ and anti-$Ca_V3.3$ gave quite specific signals that were competed by the corresponding antigen in the cells expressing the matching channel isoform. Anti-$Ca_V3.1$ from Santa Cruz had some cross reaction with $Ca_V3.3$ and was competed by its corresponding antigen in HEK-293 cells expressing $Ca_V3.1$ or $Ca_V3.3$. Anti-$Ca_V3.1$ from Alomone was specific for HEK-293 cells expressing $Ca_V3.1$. In addition, the specificity of the antibodies was evaluated in mouse and human sperm using antibodies preadsorbed with the synthetic antigenic peptides (1:2–1:8 antibody:peptide ratios). In human sperm antibodies were further tested for specificity by showing that only the corresponding peptide antigen, and not the others, competed antibody binding.

2.2.2. Spermatogenic cells. Aliquots of mouse spermatogenic cells were obtained and processed as described previously [22,23]. After fixation with 5% paraformaldehyde and permeabilization with 0.1% Triton X-100, cells were incubated overnight with primary T-type Ca^{2+} channel polyclonal antibodies (1:100 dilution) and then for 1 h with Alexa 594-conjugated secondary antibodies (1:100; Molecular Probes).

2.2.3. Mouse sperm. Cells were collected from CD1 mouse epididymis. Sperm were separated into two groups: (i) non-capacitated, handling cells in non-capacitating medium (Medium 199 without bovine serum albumin, HCO_3^- and Ca^{2+}), and (ii) acrosome-reacted, where sperm after capacitation (30 min at 37°C in capacitating medium) were incubated with the Ca^{2+} ionophore A23187 (15 μM). Aliquots of these cells were fixed with 5% paraformaldehyde, permeabilized with 0.1% Triton X-100 and incubated overnight at 4°C with primary antibodies at 1:100 dilution. Lastly, samples were incubated for 1 h with the Alexa 594-conjugated antibody (1:100). Images were acquired in a confocal microscope using Comos 7.0 and analyzed using Confocal Assistant software 4.02 (Bio-Rad Microscience).

2.2.4. Human sperm. Semen was obtained from normal, fertile volunteers by masturbation after at least 2 days of abstinence. Samples were subjected to swim-up preparation (see below) and used for indirect immunofluorescence analysis as described above for mouse sperm. Control experiments were also processed as indicated for mouse samples (corresponding peptides at 1:4, 1:2 and 1:8 ratios were used for anti-$Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$, respectively). HVA channel antibodies against $Ca_V1.2$, $Ca_V1.3$, $Ca_V2.1$, $Ca_V2.2$, and $Ca_V2.3$ were from Alomone Labs, and they were diluted 1:100 (corresponding peptides were used at 1:2 ratio, except for the $Ca_V1.3$ peptide that was used at 1:4).

2.2.5. Immunogold transmission electron microscopy. Human sperm were fixed in a 4% paraformaldehyde–0.5% glutaraldehyde solution in 100 μM cacodylate buffer (pH 7.3) for 2 h and stored until processed. Cells were dehydrated in 20% ethanol for 20 min and then in 40–100% ethanol. The final pellet was embedded in LR-White resin for 5 days at 50°C. Ultrathin sections were mounted on nickel grids. Samples were first incubated in a blocking solution containing 10% fetal bovine serum (FBS) and 0.005% Tween 20, and then incubated overnight with Ca_V3 channel primary antibodies diluted 1:25 in phosphate-buffered saline–3% FBS. Subsequently, samples were incubated for 1 h with a rabbit anti-goat IgG secondary antibody (1:30) conjugated to 10 nm colloidal gold particles (Sigma). The sections were counterstained with uranyl acetate and lead citrate and then examined using a Jeol 100SX electron microscope.

2.3. Electrophysiology

Ca^{2+} currents were recorded in spermatogenic cells using the whole-cell configuration of the patch-clamp technique as described previously [13]. Briefly, cells were bathed in a solution containing (in mM): $CaCl_2$ 10; NaCl 130; KCl 3; $MgCl_2$ 2; $NaHCO_3$ 1; NaH_2PO_4 0.5; HEPES 5; glucose 10 (pH 7.3). The internal solution consisted of (mM): $CsMeSO_3$ 110; CsF 10; CsCl 15; $CaCl_2$ 4.6; EGTA 10; HEPES 5; ATP-Mg_2 4; phosphocreatine 10 (pH 7.3). Currents were sampled at 10 kHz and recorded (following filtering 5 kHz) by a patch-clamp amplifier (Axopatch 200A; Axon Instruments) interfaced to a personal computer via an A-to-D board (DigiData 1200A; Axon) using computer-driven software (pCLAMP 6.0.3; Axon). Capacity transients were electronically compensated, and linear leak and residual capacity currents were subtracted on-line using a P/4 protocol.

2.4. Evaluation of human sperm motility

After liquefaction, 1 ml of Ham's F-10 was applied to 1 ml of semen to allow the motile sperm to swim up into the upper layer of the suspension (1 h at 37°C). Swim-up sperm were collected, adjusted to $8–12\times10^6$ cells/ml and their motility determined as previously described [19]. For each sample, ~1000 motile sperm were tracked and analyzed with the Hamilton-Thorne HTM-IVOS-12 CASA system. Sperm motility parameters measured included progressive velocity (VSL), path velocity (VAP), and curvilinear velocity (VCL), as well as amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN = VSL/VCL × 100%) and straightness (STR = VSL/VAP × 100%). Ca^{2+} channel antagonists were prepared as 100 mM stock solutions in deionized water (mibefradil and calciseptine) or dimethylsulfoxide (gossypol and nifedipine).

3. Results and discussion

Mouse spermatogenic cells functionally express mainly T-type currents, and RT-PCR studies have indicated the expression of two different genes encoding T-type channels ($Ca_V3.1$ and $Ca_V3.2$) in both mouse and human male germ cells [13–15]. However, the expression of the third member of the Ca_V3 family had not been determined. Our PCR experiments using specific primers generated a band of 404 bp, which was confirmed by sequencing to be a fragment of the mouse $Ca_V3.3$ channel (Fig. 1A). The RNA expression of this isoform had been shown to be restricted to the brain [12,25], but recently its presence was also found in human ejaculates [16].

Given that mRNAs for the three T-type Ca^{2+} channels are present in mouse spermatogenic cells, it became necessary to establish whether the proteins are also expressed in these cells and in mature sperm. To this end, specific polyclonal antibodies against both N- and C-terminal sequences of Ca_V3 channels were used. Most of the results shown in both immature germ cells and mature sperm were obtained with antibodies against the C-terminus, however qualitatively similar findings were observed with the N-terminus antibodies. Con-

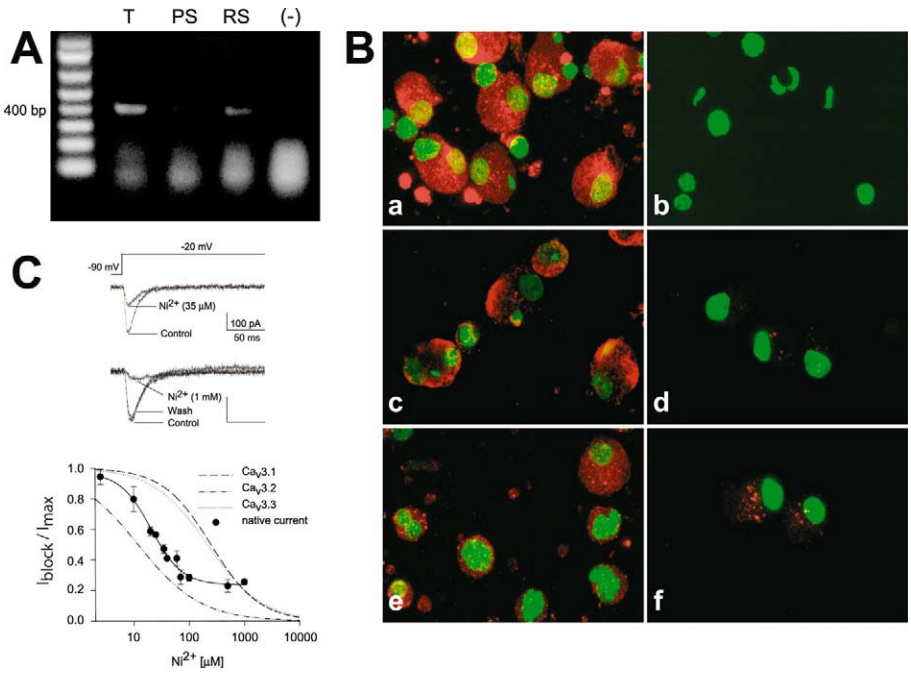

Fig. 1. Ca_V3 proteins are expressed in spermatogenic cells. A: RNA was extracted from spermatogenic cells and subjected to RT-PCR with specific primers based on the sequence of $Ca_V3.3$. Lanes 2–4 show PCR products generated from mouse testis (T), pachytene spermatocytes (PS), and round spermatids (RS), respectively. Lane 5 is a negative control (−). The product was sequenced to verify specific amplification. Molecular weights of the standards are shown on the left. B: Representative confocal micrographs from cells immunostained with $Ca_V3.1$ (a), $Ca_V3.2$ (c) and $Ca_V3.3$ (e) channel antibodies, showing the cluster arrangement of the proteins (red), or stained with the anti-Ca_V3 antibodies preincubated with the corresponding antigen peptide to demonstrate that specific staining is blocked by the peptide (b, d, and f); nuclei are shown in green. C: Representative superimposed whole-cell patch-clamp Ca^{2+} current traces activated in mouse spermatogenic cells by 200 ms pulses to −20 mV from a holding potential of −90 mV (upper panel) before and after application of 35 μM and 1 mM Ni^{2+}. The lower panel shows the dose–response analysis of Ni^{2+} blockade. Following exposure to the indicated concentration of the inhibitor, peak current amplitude was normalized to the control. The percentage of inhibition is compared with that obtained from HEK-293 cells expressing $Ca_V3.1$, $Ca_V3.2$ or $Ca_V3.3$. Smooth curves were generated with the Hill equation according to the parameters reported from currents through the cloned channels [24]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

focal immunoimages of spermatogenic cells incubated with anti-Ca_V3 antibodies revealed a punctate pattern of fluorescence possibly indicating clusters of channels at the plasma membrane, or intracellular accumulation of channel protein (Fig. 1B). The strongest signal was observed with the $Ca_V3.1$ antibody (Fig. 1Ba). Nuclei, shown in green due to staining with SYTOX Green, excluded immunostaining. Likewise, practically no signal was detected when the antibodies were preincubated with their corresponding antigen peptide (Fig. 1Bb,d,f), demonstrating that the staining shown in panels a, c, and e is specific.

Consistent with the expression of different Ca_V3 proteins, the dose–response analysis for Ni^{2+} blockade performed on native Ca^{2+} currents in mouse spermatogenic cells revealed the presence of subtypes of low-threshold channels. Representative whole-cell patch-clamp recordings (Fig. 1C, upper panel) indicated that the IC_{50} for Ni^{2+} blockade was ~21 μM (Fig. 1C, lower panel). In contrast, as reported by Lee and colleagues [24], the Ni^{2+} IC_{50} for $Ca_V3.2$ is ~12 μM and the IC_{50} values determined for $Ca_V3.1$ and $Ca_V3.3$ are ~20-fold higher. Considering the data in recombinant channels, the potency of Ni^{2+} in spermatogenic cells is intermediate. These results, analyzed as indicated by Lee et al. [24], suggest that the native current may arise from the activity of different types of low-threshold channels, with $Ca_V3.2$ contributing around 60%.

We next examined the expression of Ca_V3 channels in mature mouse sperm. Although our results for $Ca_V3.1$ were inconclusive with the anti-α_{1G} antibody from Santa Cruz, the use of a new polyclonal anti-$Ca_V3.1$ antibody (Alomone Labs) corroborated specific immunostaining in the head (50% of sperm) and principal flagellar piece (Fig. 2A). Fig. 2B also shows illustrative confocal images before (left) and after induction of the sperm AR with a Ca^{2+} ionophore (right). Panel Ba illustrates that $Ca_V3.2$ immunoreactivity was confined to the head overlying the acrosome. Upon AR the $Ca_V3.2$ signal did not undergo redistribution and remained associated with the head. However, this process decreased fluorescence staining giving a vesiculated pattern (panel Bb) consistent with the formation of hybrid vesicles [26]. Panel Bc shows that the $Ca_V3.3$ channel is located in the flagellar midpiece and not in the head or the principal piece of the tail. As anticipated its distribution was unchanged in acrosome-reacted sperm (panel Bd). Controls discussed in Section 2 using anti-Ca_V3 antibodies on HEK-293 cells separately expressing the three Ca_V3 channel isoforms indicate that the staining illustrated in Fig. 2 is specific.

Thereafter we sought to determine the expression of T-type channels in human sperm. As in the case of mouse sperm, expression of $Ca_V3.1$ in human sperm could not be confidently determined with the Santa Cruz antibody since immunostaining was not fully blocked by the corresponding antigen peptide. However, anti-$Ca_V3.1$ from Alomone gave specific binding in the head as well as in the mid and principal pieces of the tail (Fig. 3Aa). The $Ca_V3.2$ antibody recognized the principal piece with an intense punctate signal and excluded

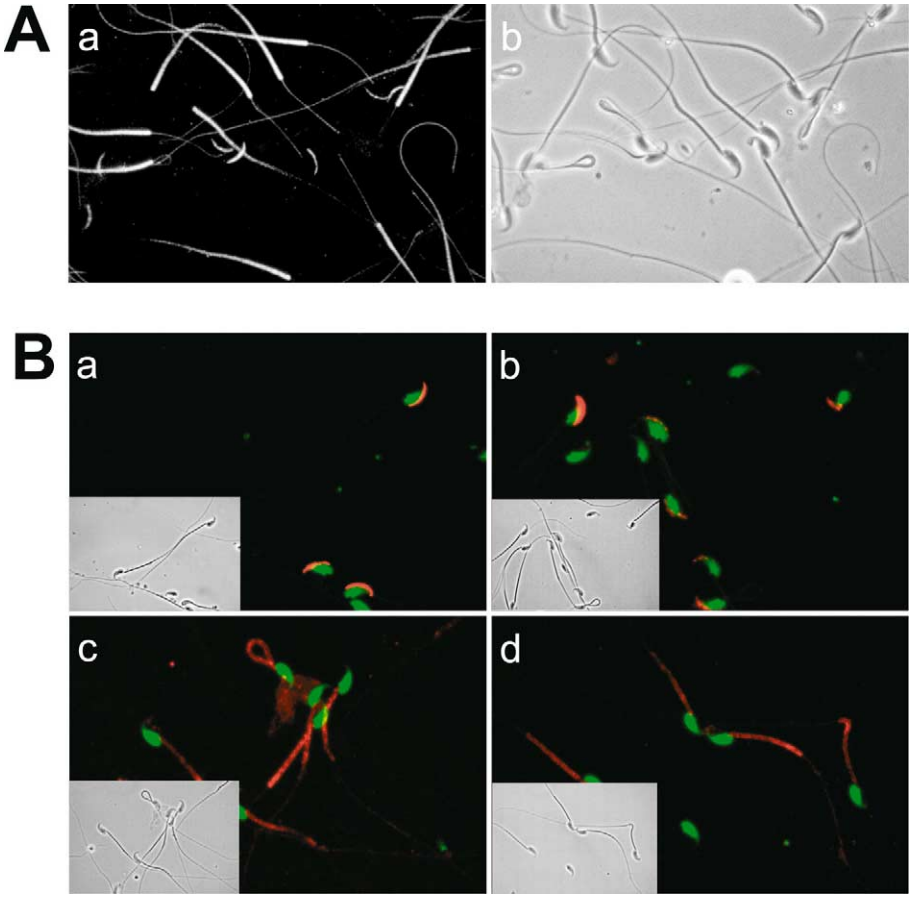

Fig. 2. Ca_V3 proteins are expressed and differentially localized in mouse epididymal sperm. Cells were attached to glass slides, and subjected to indirect immunofluorescence. A: Shown are confocal immunofluorescence images of mature sperm stained with anti-$Ca_V3.1$ antibodies (panel a). Panel b shows the corresponding phase contrast image. B: Ca_V3 confocal immunofluorescence images in mouse sperm before and after undergoing the AR. Paired phase contrast (inset) and fluorescence micrographs of anti-$Ca_V3.2$ and $Ca_V3.3$ in acrosome-intact (a, c) and acrosome-reacted sperm (b, d). Nuclei are shown in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the end piece of the flagellum (Fig. 3Ab). In addition, a faint $Ca_V3.2$ immunofluorescent signal was noted in the base of the human sperm head. On the other hand, the fluorescent signal for $Ca_V3.3$ (N-terminal antibody) was restricted to the midpiece of the flagellum (as in mouse sperm) (Fig. 3Ac). Binding of the Ca_V3 antibodies was competed only by the corresponding peptide antigen but not by the other peptides (not shown), thus indicating their specificity. To our knowledge this is the first evidence for the localization of Ca_V3 channels in human sperm.

Ca_V3 protein expression and localization in human sperm was corroborated using transmission electron microscopy. Ultrathin sections from embedded cells were incubated with the Ca_V3 antibodies followed by a secondary antibody conjugated to colloidal gold. Control sections showed only background levels of gold particles with no specific sperm localization. The micrographs in Fig. 3B show that gold particles were preferentially associated with tail plasma membrane, less in the head and rarely in the cell interior. The lower abundance of Ca_V channel in the head and specifically $Ca_V3.1$ found with this technique may help to explain our difficulty to detect these channels by immunofluorescence.

Given that Ca_V3 channels are present in the human sperm flagella, we tested whether two T-type channel inhibitors, mibefradil [11,27,28] and gossypol [18], influence motility. To this end, a post-swim-up sperm suspension was diluted in culture medium in the absence or presence of different concentrations of the channel antagonists and motility was analyzed using CASA. Interestingly, concentrations of these two compounds that inhibit Ca_V3 channels only reduced basal motility by $< 3\%$ after 1 min, in agreement with our previous findings with low Ni^{2+} concentrations (50 μM) that block these channels [19]. These observations indicate that Ca_V3 channels essentially do not influence the basal motility of human sperm. However, higher doses of mibefradil (30 μM) and gossypol (40 μM) that block HVA channels decreased human sperm velocity parameters making their trajectories less progressive (Table 1). These results may explain, at least in part, why

Table 1
Effects of Ca_V channel antagonists on human sperm motility

CASA parameter	Mibefradil 30 μM (% inhibition)	Gossypol 40 μM (% inhibition)
Basal motility	25 ± 8	21 ± 6
VAP	33 ± 9	23 ± 6
VSL	31 ± 10	27 ± 5
VCL	31 ± 5	13 ± 8

Data are expressed as mean ± S.E.M. ($n = 3$–8 separate determinations; three different donors).

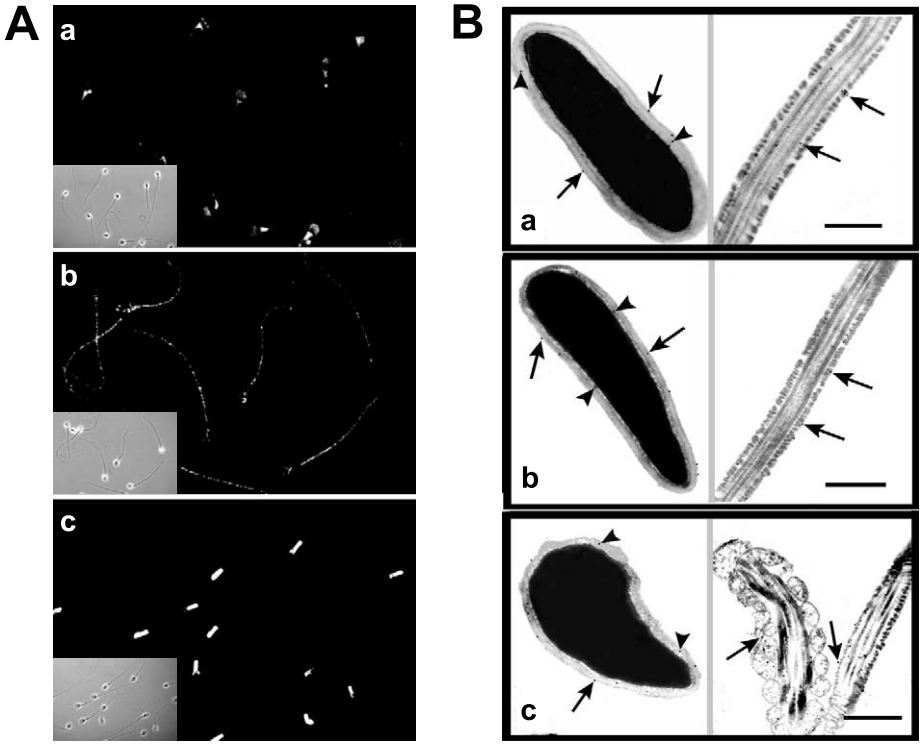

Fig. 3. Ca_V3 proteins are differentially expressed in human sperm. A: Representative confocal micrographs from cells stained with $Ca_V3.1$- (a), $Ca_V3.2$- (b) and $Ca_V3.3$- (c) specific antibodies showing the immunofluorescence localization of the proteins. Insets represent the corresponding phase contrast images. B: Immunogold electron microscopy detected Ca_V3 channels in human sperm. Ultrathin sections of human sperm were incubated with $Ca_V3.1$ (a), $Ca_V3.2$ (b) and $Ca_V3.3$ (c) antibodies. Gold particles (arrows) are associated with the plasma membrane of the sperm head (left panel of each figure). In longitudinal flagellar sections, gold labeling is observed associated with the plasma membrane as well as in the ribs of the fibrous sheath at the principal piece (right panel of each figure). $Ca_V3.3$ antibody labeled the mitochondrial sheath at the midpiece of the flagellum (c). Scale bar = 0.5 μm.

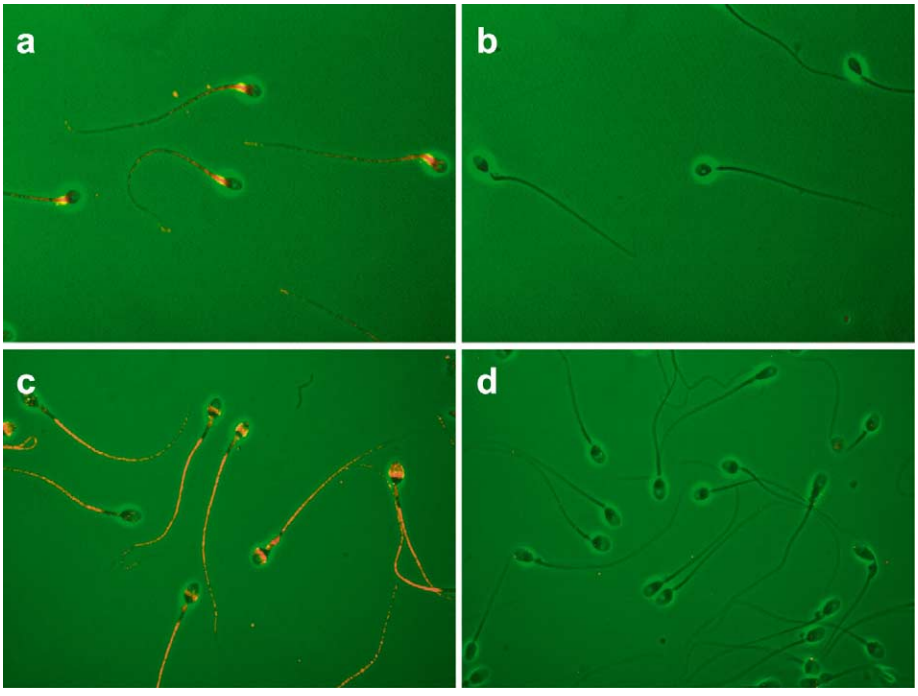

Fig. 4. $Ca_V1.2$ and $Ca_V2.3$ channels are present in human sperm. Representative confocal micrographs from sperm cells immunostained with $Ca_V1.2$ antibodies (a) showing strong specific staining at the mid and the end pieces of the tail, as well as with $Ca_V2.3$ antibodies (c) showing signal in the principal piece of the flagellum and the equatorial segment of sperm head. Panels b and d are control experiments in which the antibodies were preincubated with the corresponding antigen peptide to demonstrate specificity.

gossypol and possibly mibefradil, like other Ca_V channel antagonists, can affect human sperm fertilizing ability [29,30].

The previous results also suggest the possible presence of HVA channels in human sperm. $Ca_V1.2$ and $Ca_V2.1$ are present in the tail of mouse sperm [21,22,31] and $Ca_V1.2$ in the acrosomal region of human sperm [32]. In our hands, specific antibodies for these channels revealed positive immunostaining for $Ca_V1.2$ strong in the midpiece and less intense in the principal piece (Fig. 4a), and for $Ca_V2.3$ in the principal piece and the equatorial segment of the head (Fig. 4b). Antibodies against $Ca_V1.3$, $Ca_V2.1$ and $Ca_V2.2$ did not produce specific signals. In spite of the presence of HVA channels in the human sperm flagella, two other blockers of Ca_V1 channels, nifedipine (up to 20 μM) and calciseptine (up to 5 μM) [33], did not significantly affect motility, ruling out an important role of Ca_V1 channels in this function. On the other hand, recently it was reported that a transgenic mouse lacking $Ca_V2.3$ had motility alterations [34].

In conclusion, we identified the expression of $Ca_V3.3$ mRNA in mouse male germ cells, an isoform not previously described in these cells. This study also provides what is to our knowledge the first evidence that all three types of Ca_V3 channels are detected in male germ cells and display differential distribution in sperm. After differentiation and maturation $Ca_V3.1$ and $Ca_V3.2$ channels are present on the mouse sperm head, indicating that they may participate in regulating acrosomal exocytosis. Recently mice deficient in $Ca_V3.2$ were found to be fertile, which suggests $Ca_V3.1$ might be enough to allow the sperm AR [36]. Interestingly, Ca_V3 channel activity, particularly $Ca_V3.2$, has been associated with the AR in human sperm [14,35]. Likewise, tail localization of Ca_V3 channels suggested they may influence sperm motility. This was also the case for $Ca_V1.2$ and $Ca_V2.3$ channels. However, blockers of Ca_V1 and Ca_V3 channels did not significantly affect basal motility, indicating they do not play a major role in this sperm function. The possibility that these channels participate in hyperactivated motility, as Catsper does [21], cannot be ruled out.

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