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Veratridine-sensitive Na⁺ channels regulate human sperm fertilization capacity

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Abstract

Aims: The sperm plasma membrane contains specific ion channels and transporters that initiate changes in Ca^{2+} , Na^+ , K^+ and H^+ ions in the sperm cytoplasm. Ion channels are key regulators of the sperm membrane potential, cytoplasmic Ca^{2+} and intracellular pH (pH_i), which leads to regulate motility, capacitation, acrosome reaction and other physiological processes crucial for successful fertilization. Expression of epithelial sodium channels (ENaC) and voltage-gated sodium channels (Na_v) in human spermatozoa has been reported, but the role of Na^+ fluxes sodium channels in the regulation of sperm cell function remains poorly understood. In this context, we aimed to analyze the physiological role of Na_v channels in human sperm.

Main methods: Motility and Hyperactivation analysis was conducted by CASA analysis. Flow cytometry and spectrophotometry approaches were carried out to measure Capacitation, Acrosome reaction, immunohistochemistry for Tyr-residues phosphorylation, $[\text{Ca}^{2+}]_i$ levels and membrane potential.

Key findings: Functional studies showed that veratridine, a voltage-gated sodium channel activator, increased sperm progressive motility without producing hyperactivation while the Na_v antagonist lidocaine did induce hyperactivated motility. Veratridine increased protein tyrosine phosphorylation, an event occurring during capacitation, and its effects were inhibited in the presence of lidocaine and tetrodotoxin. Veratridine had no effect on the acrosome reaction by itself, but was able to block the progesterone-induced acrosome reaction. Moreover, veratridine caused a membrane depolarization and modified the effect of progesterone on $[\text{Ca}^{2+}]_i$ and sperm membrane potential.

Significance: Our results suggest that veratridine-sensitive Na_v channels are involved on human sperm fertility acquisition regulating motility, capacitation and the progesterone-induced acrosome reaction in human sperm.

Key words: Veratridine, Na_v channels, tyrosine phosphorylation, membrane potential, human sperm fertility.

Introduction

During their transit through the female genital tract sperm cells undergo a series of functional and biochemical modifications which are essential for acquisition of fertilization competence and interaction with the oocyte [1]. These changes include the acquisition of progressive motility, capacitation, hyperactivation and the acrosome reaction (AR) and are accompanied by changes in the membrane fluidity, stimulation of cAMP pathways, increases in tyrosine phosphorylation, intracellular Ca^{2+} and pH (pH_i) and hyperpolarization of the sperm membrane potential [2-9]. All these processes depend on the existence of a close communication between spermatozoa and the female reproductive tract [10]. It is now widely accepted that ion transport through the plasma membrane is an essential step in sperm signaling and regulate the transfer of information between sperm cells and the female genital tract [3, 9, 11-14].

Many different ion channels have been identified in the sperm cell membrane [2, 8, 15-19]. Among them, the sperm-specific Ca^{2+} channels CatSper are widely distributed in the principal piece of the flagellum and play a key role in regulating hyperactivation and AR [18, 20-23]. Molecular and functional studies have demonstrated the presence of different types of K^+ channels in spermatozoa, and activation of the sperm-specific SLO3 K^+ channel appears to be an essential mechanism for sperm membrane hyperpolarization [8, 24]. Na^+ channels, including epithelial channels of the ENaC family [14] and voltage-gated Na^+ channels (Na_v channels) [17, 25] are also present in spermatozoa and may regulate sperm function, although their role remains poorly understood.

Na_v channels are very complex proteins composed by one α and one or more β auxiliary subunits [26]. Nine different α subunits, ($\text{Na}_v1.1$, $\text{Na}_v1.2$, $\text{Na}_v1.3$, $\text{Na}_v1.4$, $\text{Na}_v1.5$, $\text{Na}_v1.6$, $\text{Na}_v1.7$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$) and four β subunits (β_1 , β_2 , β_3 and β_4) have been cloned in mammals, each one encoded by a different gene [26]. We have recently reported that the mRNAs that encode the different $\text{Na}_v \alpha$ subunits ($\text{Na}_v1.1$ -1.9) and three of the four auxiliary β subunits (β_1 , β_3 and β_4) are expressed in human testis and mature spermatozoa [17]. Immunofluorescence studies showed that Na_v channel proteins are present in sperm cells and show specific and different localizations [17, 25]. Moreover, the Na_v channel activator veratridine caused a high increase in sperm progressive motility, a response that was accompanied by little (capacitated spermatozoa) or no increase (uncapacitated spermatozoa) in intracellular free Ca^{2+} levels, $[\text{Ca}^{2+}]_i$ [17, 25].

The effects of veratridine were partially mediated by activation of $\text{Na}_v1.8$, and involved activation of additional Na_v channels, supporting a role for these proteins in the regulation of mature sperm function [25]. However, their precise function is still far to be known. The aim of the present study was to characterize more deeply the role of Na_v channels in the regulation of processes essential for the fertilization competence of human sperm cells such as motility, capacitation and the progesterone-induced acrosome reaction.

Materials and methods

Physiological solutions

The physiological salt solution (PSS) used as sperm capacitating medium consisted of NaCl 101.6 mM; KCl 4.69 mM; CaCl₂ 2.0 mM; NaHCO₃ 25 mM; MgSO₄ 0.2 mM; KH₂PO₄ 0.4 mM; Glucose 2.78 mM; HEPES 10 mM; sodium pyruvate 0.33 mM; sodium lactate 21.4 mM; bovine serum albumin (BSA) 5 mg/ml; pH adjusted to 7.4. Bicarbonate-free medium was prepared by replacing NaHCO₃ with NaCl.

Semen samples and sperm preparation

This study was approved by the Ethics Committee of CSIC and all donors gave written informed consent. Freshly ejaculated semen was collected from 98 healthy donors (18-35 years old) after 3-4 days sexual abstinence. Samples were allowed to liquefy at 37°C for 30 min and examined. Only those samples with normal sperm parameters, according to the World Health Organization (WHO) guidelines, were included in the study. Spermatozoa were separated from seminal plasma by a discontinuous density gradient (Spermgrad-125, Vitrolife, Kungsbacka, Sweden), and then capacitated by incubation for 2 h at 37°C in 5% CO₂ for subsequent experiments.

Human sperm motility studies

Spermatozoa were capacitated and adjusted to a concentration of 50 x 10⁶ cell/ml. Motility analysis was conducted by computer-assisted sperm analysis (CASA) (Sperm Class Analyzer, S.C.A., Microptic, Barcelona, Spain) as described previously [17, 25]. Following WHO guidelines, the motility pattern of sperm cells was defined as: progressive motility (A + B grade), non-progressive motility (C grade) and immotility (D grade).

To investigate the effects of veratridine, individual sperm samples were divided into four aliquots, two of them were maintained in bicarbonate-containing medium and the other pair was incubated in bicarbonate-free medium. One aliquot of each pair was treated with a single concentration of veratridine and the other with the corresponding solvent (time-matched paired controls). The concentration of veratridine (10 µM) was chosen from previous experiments [17]. Sperm motility was measured 5 min before addition of veratridine or its solvent (initial value at time 0) and after a contact time of 2, 15, 30 and 60 min. Values of sperm progressive motility, non-progressive motility and immotility were expressed as the positive or negative percentage increment in

motility produced by the drug relative to the value observed at the same time in solvent-treated time-matched paired controls (Δ sperm motility).

Analysis of sperm hyperactivation

Hyperactivation was measured by CASA system following previously described procedures [25]. Sperm samples (50×10^6 cell/ml) were incubated with veratridine (10 μ M), with the broad-spectrum antagonist of Na_v channels lidocaine, (0.1, 1 and 5 mM) or with the corresponding solvent (time-matched paired controls). Hyperactive motility was measured after 15, 30 and 60 min. Values were expressed as the percentage of hyperactivated spermatozoa in each sample.

Protein tyrosine phosphorylation measurement

Evaluation of sperm capacitation was performed by analysis of tyrosine phosphorylation (PTyr) status by immunocytochemistry and flow cytometry in PSS, as has been previously reported [2, 13, 27]. Briefly, uncapacitated sperm samples were adjusted to a concentration of 10×10^6 cells/ml and divided into different aliquots, which were incubated at 37° in 5% CO_2 . Dose-response curves were performed by incubating sperm aliquots with veratridine (1, 3, 10 or 30 μ M) or solvent for 30 min. In subsequent experiments, aliquots were untreated (time-matched paired controls) or treated with veratridine (10 μ M) or its solvent for 30, 60, 120, 180 min and 25 h. In a third set of experiments, the effect of veratridine on PTyr was analyzed in the presence of tetrodotoxin (TTX, 0.1 mM) or lidocaine (5 mM) or the corresponding solvent, which were added 10 min before veratridine. At the times indicated, spermatozoa were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 for 10 min at room temperature. For immunofluorescence detection, samples were incubated with a mouse monoclonal antibody designed to recognize human tyrosine phosphoproteins and conjugated with Alexa Fluor 488 (sc-508 AF488, Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:200 dilution for 1 h. For immunocytochemistry analysis, sperm cells were washed and smeared onto poli-L-lysine coated slides. Slides were mounted using Prolong Gold antifade reagent (Invitrogen, Molecular Probes, Eugene, OR) and examined with a Olympus BX-51 fluorescence microscopy (Tokyo, Japan). For flow cytometry analysis, fluorescence data from at least 20,000 events per sample were

captured on a BD Accuri C6 flow cytometer (BD Biosciences, San José, CA). Green fluorescence was collected in the FL1 sensor and analyzed using CFlow Plus software. Fluorescence values at each time (F) were expressed as the positive or negative increment in the percentage of PTyr-positive sperm cells (F-F0) or normalized versus PTyr-positive values at time 0 $((F-F0/F0) \times 100)$.

Viability assay

The effects of veratridine on sperm vitality were analyzed in parallel to the study of its effects on Tyr phosphorylation and at the same times (30 min -24 h) by an eosin-nigrosin assay. The percentage of viable sperm (sperm head unstained indicating living sperm) and non-viable sperm (sperm head stained indicating dead spermatozoa) was assessed by means of light microscopy in aliquots untreated (controls) or treated with veratridine or its solvent and counting a minimum of 200 spermatozoa. Replicate counts of 200 sperm were performed on two different slides and sperm viability was expressed as a percentage of total sperm cells.

Human AR measurement

Capacitated spermatozoa were incubated at 37°C under 5% CO₂ with veratridine (10 µM) or its solvent (paired controls) during 1 hour. After that, veratridine- and solvent-treated sperm cells were co-incubated with progesterone (P4, 1 µM) during the last 15 min of treatment and AR measured as reported previously [27-29]. To ascertain the specificity of the alkaloid we co-incubate samples in the presence of Na_v Channels antagonist lidocaine (5 mM) or its solvent, which were added to the sample 10 min before veratridine. Fluorescein isothiocyanate (FITC)-conjugated anti-CD46 (1 hour at room temperature) and Hoechst 33258 (2 min, room temperature) were used as AR molecular marker and viability dyes, respectively. Fluorescence data from at least 100,000 events was analyzed in a flow cytometer (FACScalibur, Becton Dickinson, San Jose, CA, USA). Green fluorescence from anti-CD46 and blue fluorescence from Hoechst 33258 were collected in the FL1 and FL9 sensor, respectively. Fluorescence from live spermatozoa were determined by subtraction of dead sperm cells background fluorescence in each histogram. Histograms were analyzed using the Summit v4.3 software and percentage of CD46 positive cells was measured.

Measurement of sperm intracellular Ca²⁺, [Ca²⁺]_i

[Ca²⁺]_i measurements were performed following previously published procedures [17, 25]. Capacitated spermatozoa were adjusted to a concentration of 10 x 10⁶ cell/ml and incubated with the acetoxymethyl ester form of Fura-2 (Fura-2/AM, 8 μM, Molecular Probes, Invitrogen, Eugene, OR, USA) for 60 min at room temperature in the presence of the noncytotoxic detergent pluronic acid (0.1%, Molecular Probes). After loading, the cells were washed, resuspended in PSS solution—and used within the next 2–7 hours. Sperm aliquots (1 ml) were placed in the quartz cuvette of a spectrofluorometer (SLM Aminco-Bowman, Series 2, Microbeam, Barcelona, Spain) and magnetically stirred at 37°C. The sperm suspension was alternatively illuminated with two excitations wavelengths (340 nm and 380 nm) and the emitted fluorescence was measured at 510 nm. Changes in [Ca²⁺]_i were monitored using the Fura-2 (F340/F380) fluorescence ratio as described previously [17, 25].

The effects of veratridine or its solvent in [Ca²⁺]_i were measured in sperm cells maintained in bicarbonate-containing solution or washed extensively and incubated in bicarbonate free-medium for 15 min before addition of veratridine or its solvent. In another set of experiments, we analyzed the [Ca²⁺]_i changes induced by P4 (1 μM) in sperm aliquots untreated or pretreated with veratridine or its solvent. In these experiments, sperm suspensions were maintained in PSS and P4 was added to the bath 10 min after addition of veratridine or its solvent. Calibration of [Ca²⁺]_i was achieved according to the equation of Grynkiewicz [30] adding 20 μl of Triton X-100 (5%), to obtain the maximal response, followed by addition of 100 μl of EGTA (40 mM) to obtain the minimal response.

Measurement of sperm membrane potential

For measurement of changes in membrane potential, sperm suspensions (15 x 10⁶ cell/ml) were capacitated and then exposed to the potential sensitive fluorescent dye DIBAC₄(3), which was added at a final concentration of 2 μM. Fluorescence was then monitored essentially as described for [Ca²⁺]_i measurement, using an excitation wavelength of 490 nm and an emission wavelength of 517 nm. Mitochondrial membrane potential was dissipated by adding carbonyl cyanide m-chlorophenylhydrazone (CCCP, 0.5 μM). After adding the dye to the sperm suspension fluorescence decreased, reaching equilibrium within 5-10 min. We then measured the effects of veratridine, P4 (1 μM) or the corresponding solvent and also analyzed the effects of P4 in sperm suspension aliquots pretreated with veratridine or its solvent. KCl

(80 mM) was added to the cuvette at the end of each experiment and changes in membrane potential were expressed relative to the maximal response induced by KCl.

Statistical analysis

Values (means $\pm$ SEM) were obtained by pooling individual data and n represents the number of experiments in sperm samples from n different donors. Statistical analyses were performed using Mann–Whitney’s U (for comparison of mean ranks between two groups) or Kruskal–Wallis followed by Dunn’s (to compare more than two groups) non-parametric tests. These procedures were undertaken using GRAPHPAD PRISM (version 5.0) program. $P < 0.05$ values were considered significant.

Results

Effects of veratridine on sperm motility in NaHCO_3 -containing and NaHCO_3 -free solution

The incubation of sperm cells in a bicarbonate-free medium caused a great reduction of progressive motility that was observed after 2 min incubation and maintained for 60 min (Figure 1A). Veratridine (10 μM) induced a quantitatively similar increase in sperm progressive motility in both bicarbonate-containing and bicarbonate-free medium that was also maintained during the time of the experiment (Figure 1B). The veratridine solvent did not modify motility in the absence or in the presence of bicarbonate in the medium (data not shown).

We next evaluated the effect of veratridine on sperm $[\text{Ca}^{2+}]_i$ levels in the presence and absence of bicarbonate in sperm suspensions loaded with Fura-2. In a bicarbonate-containing medium veratridine caused a small transient increase in $[\text{Ca}^{2+}]_i$ that decline slightly and was followed by a prolonged plateau. In sperm suspensions incubated in bicarbonate-free medium, however, veratridine did not induce any $[\text{Ca}^{2+}]_i$ mobilization (Figure 1C).

Effects of veratridine on sperm hyperactivation

Veratridine failed to induce hyperactivated motility in spermatozoa, confirming our previous results [25] (Supplementary Figure 2). In contrast, the Na_v antagonist lidocaine (0.1-5 mM) increased the percentage of hyperactivated sperm and the effects were significant at the concentration of 5 mM (Figure 2). This lidocaine concentration was therefore used in subsequent experiments.

Effects of veratridine on sperm protein tyrosine phosphorylation

Due to the fact that tyrosine phosphorylation is an event that occurs during sperm capacitation (2), we analyzed PTyr levels by immunofluorescence and flow cytometry. Immunofluorescence studies revealed PTyr-positive immunoreactivity at the flagellum of sperm cells (Figure 3A). Veratridine (10 μM) caused an increase of the PTyr signal in the sperm cells after 30 min treatment (Figure 3A). Flow cytometry analysis confirmed that veratridine (1-30 μM) caused a concentration-dependent increase in the percentage of PTyr-positive cells and the maximal response was reached with 10 μM (Fig 3B, not shown for 1 μM). Evaluation of the time course of the response to veratridine 10 μM showed that the alkaloid induced a time-dependent, progressive

increase of PTyr in sperm samples (Figure 3C, Figure S1). After 30 min incubation, PTyr levels were significantly higher in the presence of veratridine ($P<0.05$, $n=30$, Figure 3B,C) while the veratridine solvent induced no change from the values observed in untreated control samples during the time period studied (Figure 3C). The veratridine-induced increase in PTyr was also observed after incubation for 60 and 120 min, but there was a tendency towards a lower effect at prolonged times, due to the time-dependent increase in PTyr that occurred in control samples (Figure 3C and Figure S1). In fact, after 3 and 25 h incubation, the percentage of PTyr-positive cells in sperm samples treated with veratridine were not significantly different from the values in the time-matched paired control aliquots, either untreated or treated with the veratridine solvent (Figure 3C). The response to veratridine was inhibited in the presence of TTX (0.1 mM) and lidocaine (5 mM) (Figure 4A,B).

Veratridine did not modify sperm vitality and the percentage of living and death cells was similar to that observed in solvent-treated aliquots (Figure 4C)

Effect of veratridine on human sperm P4-induced AR

Flow cytometry analysis showed that veratridine did not modify the percentage of CD46-positive sperm cells after 1 hour incubation, compared to solvent-treated paired controls (DMSO) (Figure 5A). The Na_v antagonist lidocaine (5 mM), however, increased the percentage of acrosome reacted sperm ($P<0.05$, $n=10$, Figure 4 A). P4 induced the AR, measured as an increase in the percentage of CD46-positive cells and veratridine diminished this effect. In fact, we observed a significant reduction in the percentage of CD46-positive cells with respect to P4 alone when sperm cells were incubated together with both veratridine and P4 ($P<0.01$, $n=10$, Figure 4 A). Thus, veratridine inhibited the P4-induced AR and the blockade of Na_v channels by lidocaine induced a reversion of this effect ($P<0.05$, $n=10$, Figure 5A).

Effects of veratridine and P4 on sperm $[\text{Ca}^{2+}]_i$

To further investigate the effects of veratridine on human sperm, we evaluated the effect of veratridine on the P4-stimulated $[\text{Ca}^{2+}]_i$ response. The addition of P4 caused the typical biphasic Ca^{2+} increase consisting in a rapid transient response followed by a plateau phase (Figure 5B). Veratridine modified the $[\text{Ca}^{2+}]_i$ response to P4, affecting both the peak and the plateau of the response (Figure 5B). The time to peak and down and the duration of the peak stimulated by P4 were higher in the presence of veratridine and, at the same time, there was an increase in the amplitude at the inflection and at the

plateau phase (Figure 5B,C; supplementary Table S1). This results in a broader signal and an increase in the total area of the P4 response in the presence of veratridine (supplementary Table S1).

Effects of veratridine and P4 on sperm membrane potential (E_m)

In capacitated sperm samples loaded with the potential sensitive dye DIBAC₄(3), veratridine (10 μ M) caused a rapid depolarization (15.8 ± 2.2 % of the maximal response to KCl) followed by a slow phase of decay. Veratridine solvent, however, caused a non-significant small hyperpolarization (Figure 6A,B). P4 (1 μ M) induced a rapid transient depolarization (23.7 ± 1.4 % of KCl response) followed by a repolarization phase during which the membrane potential became more negative than it was initially, a fact that occurred in all sperm samples assayed (-17.2 ± 4.4 % of the KCl response). This hyperpolarization was followed by a slow return to less negative potentials, reaching a steady-state level after approximately 200 sec (-12.2 ± 3.5 % of the KCl response). Veratridine highly modified the effect of P4 on sperm membrane potential. In the presence of veratridine, P4 induced a depolarization of higher magnitude and duration, the repolarization phase was delayed and the early and late hyperpolarization phases were inhibited (Figure 6). The area of the P4-induced depolarizing phase was 5.7 ± 1.0 fold higher in the presence of veratridine than in the presence of veratridine solvent ($P < 0.05$).

Discussion

Ion channels, including Ca^{2+} , K^{+} and anion channels are widely distributed in spermatozoa and play an important role in regulating sperm function [3, 5, 7, 13, 14, 19, 31]. The present findings demonstrate that veratridine-sensitive Na^{+} channels are also involved in the regulation of processes that are essential for the acquisition of fertilization competence in human sperm such as sperm motility, capacitation and the acrosome reaction.

Our previous findings showed that several voltage-gated Na^{+} channels are expressed, at the mRNA and protein levels, in human testis and mature spermatozoa [17]. We also found that veratridine, a Na_v channel activator, caused time- and concentration-dependent increases in progressive motility [17, 25]. The effects of veratridine on motility were observed in sperm samples incubated in a Ca^{2+} -containing or a Ca^{2+} -free medium and were accompanied by small or no changes in $[\text{Ca}^{2+}]_i$ [25] demonstrating that its effects are not dependent on Ca^{2+} influx. In the present study, we aimed to evaluate the effect of veratridine on progressive motility in a non-capacitating medium. Incubation in a bicarbonate free-medium caused a high reduction of progressive motility [32], but veratridine was still able to increase progressive motility in these sperm cells. In addition, veratridine caused the same quantitative increase in progressive motility in samples incubated in bicarbonate-containing or bicarbonate-free-medium (Figure 1) and induced a small $[\text{Ca}^{2+}]_i$ rise in bicarbonate-containing medium that was not observed in the absence of bicarbonate. Our results, thus, suggest that the effect of veratridine on progressive motility is mediated through both bicarbonate and Ca^{2+} -independent pathways. These results are consistent with the fact that the effects of veratridine on sperm motility are mainly independent on external Ca^{2+} and on the capacitated or non-capacitated state of sperm [17]. On the other hand, veratridine failed to induce hyperactivation (Fig S1), a motility pattern characterized by an increase in the flagellar beat amplitude and asymmetry that produces circular or helical trajectories, important to penetrate the oocyte zona pellucida [9, 33]. In contrast, lidocaine, a broad-spectrum Na_v channel blocker, caused hyperactivated motility, as has also been described for procaine [10, 34]. These results, together with previous data, reinforce the idea that Na_v channels act mainly on the mechanism that mediates "activated" or "linear" progressive sperm motility while Na_v channel blockade appears to be important for development of hyperactivation.

Our next aim was to evaluate the effect of veratridine on human sperm capacitation. In mammalian sperm, capacitation is associated with an increase in the tyrosine phosphorylation of a subset of proteins which are mainly located in the sperm flagellum [3, 13]. Veratridine caused a concentration-dependent increase in PTyr, measured by both immunocytochemistry and flow cytometry, and its effects were reduced in the presence of lidocaine and TTX. This confirms the involvement of Na_v channels in the veratridine-induced increase in PTyr. In comparison with paired solvent controls, veratridine increased the percentage of PTyr-positive sperm cells after 30 min treatment and the increase was maintained after 60 and 120 min. However, due to the time-dependent increase in PTyr that occurred in control samples, PTyr levels were similar in veratridine-treated and untreated samples after 3 and 25 h contact (Figure 3C). These data show that veratridine accelerates tyrosine phosphorylation but does not increase PTyr over the “physiological” levels observed in control samples, again supporting a role of Na_v channels in the initial steps of human sperm capacitation. It is interesting to note that ouabain, which increases $[\text{Na}^+]_i$ by inhibition of the Na^+/K^+ ATPase, also enhances PTyr [35, 36], demonstrating that changes in $[\text{Na}^+]_i$ are important for regulation of sperm function.

In vivo, the AR is placed following sperm capacitation and is essential for the interaction of sperm with the oocyte in the fertilization process. The present results confirm that veratridine did not have any effect *per se* on human AR [26]. In addition, our data show that the P4-induced AR was reduced in the presence of veratridine and this effect was reverted by the Na_v channels antagonist lidocaine. A recent report has shown that stimulation of AR by progesterone in mouse sperm is absolutely dependent on a specific P4-induced transitory increase in $[\text{Ca}^{2+}]_i$ [37]. Moreover, alterations of the P4-induced $[\text{Ca}^{2+}]_i$ signal prevent the AR in human spermatozoa [38]. Thus, to investigate further the role of veratridine on AR, we evaluated its effect on the $[\text{Ca}^{2+}]_i$ response to P4. In the presence of veratridine, the duration of the P4-induced $[\text{Ca}^{2+}]_i$ transient was increased, leading to a retard in the time to peak, and the phase of decay of the $[\text{Ca}^{2+}]_i$ transient was delayed. The broader $[\text{Ca}^{2+}]_i$ signal produced by P4 in the presence of veratridine might reflect a deregulation of the sperm $\text{Na}^+/\text{Ca}^{2+}$ balance [39, 40] and could avoid the presence of the second $[\text{Ca}^{2+}]_i$ peak to P4, necessary for the initiation of the AR, which is a tightly regulated mechanism [3, 37, 38].

It is well-documented that spermatozoa from several mammalian species exhibit a capacitation-associated plasma membrane hyperpolarization, which is necessary for the acrosome reaction to occur [15, 41-43]. Previous studies have demonstrated that Na^+ permeability participates in the establishment of sperm resting E_m [16]. So, we evaluated the effects of veratridine and P4 on sperm E_m . Our results show that veratridine caused a sperm membrane depolarization and modified the P4-induced response. In agreement with previous reports [40], we found that P4 induced a transient depolarization followed by a repolarization and a subsequent long hyperpolarization phase (Figure 5). In the presence of veratridine, the P4 depolarization phase was significantly higher, in magnitude and duration, and the hyperpolarization phase was inhibited. These and the results above suggest that activation of Na_v channels by veratridine leads to an imbalance on $\text{Na}^+/\text{Ca}^{2+}$ levels that modifies the effect of P4 on $[\text{Ca}^{2+}]_i$ and sperm membrane potential, thus inhibiting the P4-induced AR. In this context, a recent report has shown that depolarization of sperm membrane potential is a common feature of men with subfertility, an event that has been attributed to a dysfunction of K^+ channels [44]. Due to the fact that veratridine caused a sperm membrane depolarization, the changes on E_m observed in subfertile men might additionally involve a malfunction of sperm Na^+ channels, although further studies will be necessary to understand the precise role of Na_v channels in male infertility.

Conclusion

The present results support a role of Na_v channels in the correct balance of ion levels and regulation of sperm membrane potential, which are essential steps for the acquisition of sperm fertilization competence. Na_v channels seem to be specifically involved in the regulation of "activated" or "linear" progressive sperm motility, and act as a regulator of premature hyperactivation since Na_v channels blockage appears to be important for development of hyperactivation. Na_v activation also modifies the effect of P4 on $[\text{Ca}^{2+}]_i$ and inhibits the P4-induced AR, suggesting that Na_v channels could regulate the initial steps of human sperm capacitation and prevent a premature progesterone-induced AR in an inadequate place.

In summary, our results support a role of Na_v channels in the acquisition of sperm fertilizing ability during their way through the female reproductive tract. Na_v channels appear to play a more important role in the non-capacitated spermatozoa and in the initial preparation of sperm for fertilization, preventing a premature hyperactivation and AR.

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Competing Interests

L.C., F.M.P., A.C.-R., C.G.R., M.F.-S., N.P., J.I. and N.S. have nothing to declare.

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FIGURE LEGENDS

Figure 1. Effects of veratridine on human sperm motility and intracellular free Ca^{2+} , $[\text{Ca}^{2+}]_i$, in NaHCO_3 -containing and NaHCO_3 -free solution. (A) Progressive motility in NaHCO_3 -containing (white bars) and NaHCO_3 -free solution (black bars) at different times of incubation. Bars are means with SEM of 11 different experiments and represent % sperm of A + B motility grade. * $P < 0.05$, significant difference vs paired responses in NaHCO_3 -containing solution (B) Effects of veratridine (10 μM) on progressive motility (grade A+B sperm) at different times of incubation in NaHCO_3 -containing and NaHCO_3 -free solution. Bars are means with SEM of 7 different experiments and represent percentage changes in motility in samples treated with veratridine relative to the value observed at the same time in veratridine solvent-treated paired controls ^a $P < 0.05$, significant difference vs. controls in NaHCO_3 -containing solution. ^b $P < 0.05$, significant difference vs. controls in NaHCO_3 -free solution. (C) Effect of veratridine on $[\text{Ca}^{2+}]_i$ in human sperm cells loaded with Fura-2 and then incubated in NaHCO_3 -containing (red line) or NaHCO_3 -free solution (black line). The X axis shows time in seconds and the Y axis shows $[\text{Ca}^{2+}]_i$ data expressed by the F340/F380 ratio. Traces are representative of typical results obtained in 8 different experiments with each solution.

Fig. 2. Time-dependent effect of veratridine, lidocaine, or the corresponding solvent on human sperm hyperactivation. (A) Effect of veratridine (VER, 10 μM) and its solvent. (B) Effect of lidocaine (LID, 0.1, 1 and 5 mM) and its solvent. Bars are means with SEM of 13 different experiments and represent % hyperactivated sperm, measured by CASA. * $P < 0.05$, significant difference vs. hyperactivated sperm levels in paired controls treated with solvent.

Figure 3. Effects of veratridine on human sperm protein tyrosine phosphorylation (PTyr). (A) Immunofluorescence analysis of PTyr in samples treated with veratridine (10 μM) and solvent-treated controls for 30 min. There was a stronger reactivity of PTyr at the sperm flagellum in veratridine-treated samples. DNA of controls was stained with Hoechst 33258. Representative contrast-phase images are shown; $n = 5$. Scale bar, 2 μm . (B) Concentration-dependent effect of veratridine (3-30 μM) and its solvent on PTyr levels at different times of incubation, measured by flow cytometry. Bars are

means with SEM of 12 different experiments and represent PTyr-positive sperm cells normalized versus PTyr-positive values at time 0 ($(F-F_0/F_0) \times 100$). * $P < 0.05$, significant difference vs. PTyr levels in paired controls treated with veratridine solvent. ^a $P < 0.05$, significant difference vs. PTyr levels in samples treated with veratridine 3 μ M. (C) Time-dependent effect of veratridine (10 μ M) and its solvent on PTyr levels. Bars are means with SEM of 30 different experiments and represent the net increment in the percentage of PTyr-positive sperm cells ($F-F_0$). * $P < 0.05$, significant difference vs. PTyr levels at time 0. ^b $P < 0.05$, significant difference vs. PTyr levels in paired controls treated with veratridine solvent or untreated.

Figure 4. Effects of veratridine on human sperm protein tyrosine phosphorylation (PTyr) in the presence of tetrodotoxin or lidocaine. The effect of veratridine (VER, 10 μ M) after different incubation times was analysed in the presence of (A) tetrodotoxin (TTX, 0.1 mM) or its solvent and (B) lidocaine (LID; 5 mM) or its solvent. Bars are means with SEM of 6-12 different experiments and represent the net increment in the percentage of PTyr-positive sperm cells ($F-F_0$). * $P < 0.05$, significant difference vs. PTyr levels in paired controls treated with the corresponding solvent. (C) Effect of veratridine (VER, 10 μ M) on sperm vitality during the time. Bars are means with SEM of 12 different experiments and represent % live sperm measured by eosin-nigrosin assay.

Figure 5. Effects of veratridine on progesterone (P4)-induced AR and intracellular free Ca^{2+} , $[Ca^{2+}]_i$, response in human sperm. (A) Percentage of CD46-positive sperm cells measured by flow cytometry after incubation with solvent (DMSO), veratridine (VER; 10 μ M), lidocaine (LID; 5 mM), progesterone (P4; 1 μ M) and co-incubations with VER and P4, and VER, LID and P4. Normalized data as $[(\text{Treatment} - \text{Solvent}) / (\text{Progesterone} - \text{Solvent})] / 100$. Bars are means with SEM of 10 different experiments. * $P < 0.05$, significant difference vs solvent-treated paired controls; ^a $P < 0.05$, significant difference vs Progesterone-treated paired samples. (B) P4-induced $[Ca^{2+}]_i$ response in the presence of veratridine (black line) or veratridine solvent (red line). (C) Original tracing showing the effect of veratridine and progesterone on $[Ca^{2+}]_i$. The X axis shows time in seconds and the Y axis shows $[Ca^{2+}]_i$ data expressed by the F340/F380 ratio. TX = Triton X-100. Traces are representative of typical results obtained in 8 different experiments

Figure 6. Effects of veratridine and progesterone (P4) on human sperm membrane potential (Em). Membrane potential was measured in capacitated sperm cells loaded with the potential sensitive fluorescent dye DIBAC₄(3). **(A)** Typical tracing showing the effect of veratridine (10 μ M) or its solvent, and the response induced by P4 (1 μ M) in the presence of veratridine or its solvent (paired control). KCl (80 mM) was added at the end of each experiment. The X axis shows time in seconds and the Y axis shows Em data expressed by the fluorescence emission at 516 nm. **(B)** Effect of veratridine on Em and effect of P4 in the presence of veratridine or its solvent. D = P4-induced depolarization, H1 = early P4-induced hyperpolarization, H2 = late P4-induced hyperpolarization. Bars are means with SEM of 8 different experiments and represent percentage changes in Em expressed relative to the maximal response induced by KCl. *P<0.05, significant difference vs. responses in solvent-treated paired controls (n=8).

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Effect of veratridine (10 μ M) at different times of incubation on human sperm phospho-tyrosine levels (PTyr) measured by flow cytometry. **(A)** Representative histogram of negative controls performed omitting the primary antibody to exclude the autofluorescence of the sample confirms the specificity of the antibody (left). Representative histogram of basal sperm capacitation established PTyr-positive and – negative sperm cell population. **(B,C)** Effects of veratridine (10 μ M) on the PTyr levels at **(B)** 30 min and **(C)** 180 min. Representative histograms of sperm samples treated with solvent (black line) and veratridine (red line) are shown (n=30).

A

B

C

Figure 1

A

B

Figure 2

Figure 3

A

B

C

Figure 4

A

B

C

Figure 5

A

B

Figure 6