Membrane hyperpolarization during human sperm capacitation

I. López-González1,‡, P. Torres-Rodríguez1,‡, O. Sánchez-Carranza1, A. Solís-López1, C.M. Santi2, A. Darszon1, and C.L. Treviño1,*

1Departamento de Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62210, México 2Department of Anatomy and Neurobiology, Washington University School of Medicine, 660 S Euclid Ave, St. Louis, MO 63110, USA

*Correspondence address. Tel: +52-777-3-29-16-11; Fax: +52-777-3-17-23-88; E-mail: ctrevino@ibt.unam.mx

Submitted on October 30, 2013; resubmitted on February 19, 2014; accepted on April 9, 2014

ABSTRACT: Sperm capacitation is a complex and indispensable physiological process that spermatozoa must undergo in order to acquire fertilization capability. Spermatozoa from several mammalian species, including mice, exhibit a capacitation-associated plasma membrane hyperpolarization, which is necessary for the acrosome reaction to occur. Despite its importance, this hyperpolarization event has not been adequately examined in human sperm. In this report we used flow cytometry to show that a subpopulation of human sperm indeed undergo a plasma membrane hyperpolarization upon in vitro capacitation. This hyperpolarization correlated with two other well-characterized capacitation parameters, namely an increase in intracellular pH and Ca$^{2+}$ concentration, measured also by flow cytometry. We found that sperm membrane hyperpolarization was completely abolished in the presence of a high external K$^+$ concentration (60 mM), indicating the participation of K$^+$ channels. In order to identify, which of the potential K$^+$ channels were involved in this hyperpolarization, we used different K$^+$ channel inhibitors including charybdotoxin, slotoxin and iberiotoxin (which target Slo1) and clofilium (a more specific blocker for Slo3). All these K$^+$ channel antagonists inhibited membrane hyperpolarization to a similar extent, suggesting that both members of the Slo family may potentially participate. Two very recent papers recorded K$^+$ currents in human sperm electrophysiologically, with some contradictory results. In the present work, we show through immunoblotting that Slo3 channels are present in the human sperm membrane. In addition, we found that human Slo3 channels expressed in CHO cells were sensitive to clofilium (50 µM). Considered altogether, our data indicate that Slo1 and Slo3 could share the preponderant role in the capacitation-associated hyperpolarization of human sperm in contrast to what has been previously reported for mouse sperm, where Slo3 channels are the main contributors to the hyperpolarization event.

Key words: flow cytometry / human sperm / hyperpolarization / Slo K$^+$ channels

Introduction

After cell differentiation in the testis, spermatozoa continue to mature as they pass through the epididymis. In the female tract they undergo capacitation—a complex process, involving a series of physiological modifications, that prepares sperm to undergo the acrosome reaction (AR) when they encounter the egg (Jin et al., 2011). The AR is a single exocytic event that sperm need to undergo to penetrate the outer layers of the egg and to fuse with it.

Capacitation involves remodeling of the plasma membrane (which includes cholesterol removal), as well as extensive protein tyrosine-phosphorylation and increases in intracellular pH (pH$i$) and Ca$^{2+}$ ([Ca$^{2+}$]) (Aitken et al., 1986; Parrish et al., 1988; Thomas and Meizel, 1988; Baldi et al., 1991; Zeng et al., 1996; Flesch and Gadella, 2000; Lishko et al., 2010). In mouse and bovine sperm, only a subpopulation of spermatozoa (20–40%) becomes capacitated (Zeng et al., 1995), and the mechanisms involved in such selective capacitation and in eliciting the aforementioned cellular changes are far from clear. Notably, only capacitated sperm can undergo the AR and fertilize the egg.

In some species, including mouse, cow and horse, capacitation is also accompanied by a membrane potential (Em) hyperpolarization (Zeng et al., 1995; Arnout et al., 1999; Muñoz-Garay et al., 2001; Demarco et al., 2003; McPartlin et al., 2011; De La Vega-Beltran et al., 2012). The exact role of this hyperpolarization is not completely clear, but it has been explored especially in mouse sperm. In this species the resting membrane potential (Emr) of non-capacitated sperm lies between −35 and −45 mV and after capacitation this value reaches −65 mV (Espinosa and Darszon 1995; Zeng et al., 1995; Muñoz-Garay et al., 2001; Demarco et al., 2003; Hernández-González et al., 2006; Santi et al., 2010; McPartlin et al., 2011; De La Vega-Beltran et al., 2012). The function of this hyperpolarization was believed to be the removal of the inactivation from T-type voltage-dependent Ca$^{2+}$ channels (Ca$\nu$3),

† These authors contributed equally to this work.

© The Author 2014. Published by Oxford University Press on behalf of the European Society of Human Reproduction and Embryology. All rights reserved. For Permissions, please email: journals.permissions@oup.com
which could then be activated during the AR by physiological agonists (e.g. zona pellucida; ZP) (Zeng et al., 1995; Arnout et al., 1996, 1999; Liévano et al., 1996). However, recent electrophysiological evidence (Lishko et al., 2012) has questioned the presence of these Ca\textsuperscript{2+} channels in mature sperm. The patch clamp technique applied directly to mature mouse and/or human spermatozoa could only detect channels for four ions: Ca\textsuperscript{2+} channels [Catsper (mouse: Ren et al., 2001; and humans: Strunker et al., 2011; Lishko et al., 2011) and TRPM8 (mouse: Martínez-López et al., 2011)]; K\textsuperscript{+} channels [Slo3 (mouse: Martínez-López et al., 2009; Zeng et al., 2011; Mansell et al., 2014); Slo1 (humans: Mannowitz et al., 2013; Mansell et al., 2014)]; H\textsuperscript{+} channels (human Hv: Lishko et al., 2010) and Cl\textsuperscript{-} channels [cystic fibrosis transmembrane conductance regulator (CFTR) (mouse: FIGUEIRAS-FIERRO et al., 2013; and TMEM16A (humans: ORTA et al., 2012)]. Catsper, H\textsuperscript{+} channels and a putative specific Na\textsuperscript{+}/H\textsuperscript{+} exchanger (Lee, 1984, 1985; Wang et al., 2003) that are also present in sperm, can be affected by a change in Em, highlighting the importance of the hyperpolarization event. In addition, our group has recently shown that a membrane hyperpolarization is necessary to prepare mouse sperm for the AR (De La Vega-Beltran et al., 2012).

Previously, several groups demonstrated that the ZP-induced AR is impaired in mouse sperm if capacitation takes place in a medium with high extracellular K\textsuperscript{+} concentration ([K\textsuperscript{+}])\textsubscript{o}, a condition that prevents membrane hyperpolarization (Zeng et al., 1995; Arnout et al., 1999; De La Vega-Beltran et al., 2012). The molecules and mechanisms involved in membrane hyperpolarization are beginning to be understood at least for mouse sperm. Sperm cells from Slo3 null mice are unable to hyperpolarize during capacitation and display several defects including impaired motility, a bent ‘hairpin’ shape and failure to undergo the AR (Santi et al., 2011; Zeng et al., 2011). Altogether, these defects render Slo3 null male mice sterile. Chávez et al. (2013) recently showed that Slo3 is the principal channel responsible for mouse hyperpolarization during capacitation and that its K\textsuperscript{+} permeability increases three times during this process, while Na\textsuperscript{+} and Cl\textsuperscript{-} permeabilities remain practically the same. Despite the different roles that membrane hyperpolarization may play during capacitation, this Em change has not been demonstrated in human sperm. There are few reports regarding this subject. Linares-Hernandez et al. (1998) reported that the Em, of non-capacitated human sperm is around −40 mV, whereas Patrat et al. (2002) reported that capacitated sperm exhibit an Em of about −58 mV. An exploratory study using cell sorting and an Em sensitive dye reported that capacitated human sperm undergo a hyperpolarization (Brewis et al., 2000). However, none of these studies quantitatively determined the Em changes human sperm undergo during capacitation or examined their nature pharmacologically.

In the present work, we determined through flow cytometry experiments that: (i) there is a subpopulation of human sperm that undergoes membrane hyperpolarization during capacitation, (ii) such Em change is completely blocked by high [K\textsuperscript{+}])\textsubscript{o} and (iii) the hyperpolarization is partially sensitive to diverse K\textsuperscript{+} channels blockers, suggesting that two or more K\textsuperscript{+} channels, including Slo3, are involved in it. Furthermore, we observed that the heterologous expression of human Slo3 (hSlo3) channels in Chinese hamster ovary (CHO) cells hyperpolarizes their Em. The pharmacological profile of the expressed hSlo3 channels was consistent with the inhibition caused by Slo3 inhibitors of the capacitation-associated hyperpolarization in human sperm, but other K\textsuperscript{+} channels, namely Slo1 may also participate in the process.

## Materials and Methods

### Reagents

3,3′-Dipropylthiadicarbocyanine iodide (DiSC\textsubscript{3}(5)), propidium iodide (PI), Fluor-3 acetoxyethyl (AM) ester (Fluo-3 AM) and 2,7′-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, acetoxyethyl ester (BCECF) were purchased from Molecular Probes-Invitrogen, Inc. (Eugene, OR, USA). Charybdotoxin, iberiotoxin, slotoxin and tetrodotoxin were acquired from Alomone Labs (Jerusalem, Israel). LipofectAMINE, bovine fetal serum (BSA), Advanced Dulbecco’s modified Eagle’s medium and pcDNA3.1(−) vector, were obtained from Invitrogen (Carlsbad, CA, USA). Restriction enzymes were purchased from Fermentas (now Thermo Scientific). T4 DNA ligase was acquired from Thermo Scientific (Fisher Scientific, Pittsburgh, PA, USA). All other reagents were purchased through Sigma-Aldrich (St. Louis, MO, USA).

### Human sperm swim-up

Human sperm were obtained as previously described (Mata-Martínez et al., 2013). Briefly, ejaculated samples (obtained by masturbation) fulfilling the parameters established by the World Health Organization laboratory manual were used for the experiments. Semen samples were placed in an incubator at 37°C under 5% CO\textsubscript{2} for 30 min for liquefaction. Five hundred microliter portions of each liquefied semen sample was placed in glass test tubes and incubated for 1 h with 1 mL Ham’s F-10 medium carefully layered on top of the sample to avoid mixing of the phases (Ham’s F-10 medium was supplemented with 2 mM CaCl\textsubscript{2} and for capacitated sperm samples only with 5 mg/mL BSA). Seven hundred microliters (now containing motile spermatozoa) was then collected from the upper part of each tube and pooled into a single clean glass tube. The sample concentration was determined using a Makler Counting Chamber and adjusted to the required concentration using supplemented Ham’s F-10 medium. When required, sperm samples were incubated at 37°C under 5% CO\textsubscript{2} for 5–13 h to promote capacitation, non-capacitated sperm were incubated for the same amount of time but in a media lacking BSA.

### Human sperm analysis by flow cytometry

Sperm plasma membrane potential, pH, and [Ca\textsuperscript{2+}]\textsubscript{i} changes were monitored using DiSC\textsubscript{3}(5), BCECF and Fluo3-AM, respectively. After overnight incubation under either non-capacitating or capacitating conditions, samples were centrifuged at 750 g for 5 min. Sperm were resuspended in Human Sperm Medium (HSM); 120 mM NaCl, 15 mM NaHCO\textsubscript{3}, 4 mM KCl, 1.8 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM HEPES, 10 mM Na lactate, 5 mM D-glucose, 1 mM Na pyruvate; pH 7.4 and their concentration adjusted to 4 × 10\textsuperscript{6} cells/mL. The sperm were loaded with the fluorescent indicators by incubating them with 50 nM DiSC\textsubscript{3}(5), 0.5 μM BCECF or 1 μM Fluo3-AM during 30 min at 37°C under 5% CO\textsubscript{2}. For each experimental condition to be tested, 500 μL of cell suspension was placed in a cytometer tube, PI was added 5 min before collecting data. Data were recorded as individual cellular events using an FACSCannto II\textsuperscript{TM} cytometer (Becton Dickinson).

Forward scatter (FSC) and side scatter (SSC) fluorescence data were collected from 20 000 events per sample. Appropriate cytometer settings were selected for DiSC\textsubscript{3}(5), BCECF, Fluo3-AM and PI. Threshold levels for FSC and SSC were set to exclude signals from cellular debris (Fig. 1A and B). Thus, particles of cellular debris with background fluorescence signals outside these threshold values were not included in the counts of sperm calculated by the flow cytometer and the number of sperm available for study was less than the total number of events (20 000). The photo multiplier tube voltages used to record the fluorescence signals were as follows: FSC (480–500), SSC (520–550), Fluo3-AM/BCECF (500–580), PI (500–560) and DiSC\textsubscript{3}(5) (600–700). Unstained control samples were used to verify
that threshold settings were appropriate and to create the corresponding gates needed to discriminate debris from cells. Positive cells for DiSC 3(5), BCECF, Fluo3-AM and PI (to determine viability) were collected using allophycocyanine, fluorescein isothiocyanate FITC (for BCECF and Fluo3-AM) and PI filters, respectively. The following controls were used to set up compensation parameters: unstained sperm, sperm stained with DiSC3(5), BCECF, Fluo3-AM or PI-stained dead sperm (sperm suspended in 0.1% Triton X-100 in HSM and incubated 10 min at room temperature). Non-capacitated and capacitated sperm samples (3 × 10⁶ cells/mL, for each condition) were run and recorded. Data were analyzed using FACS Diva and FlowJo software (Tree Star 9.3.3). Cell debris, doublets and aggregates were excluded from analysis based on a dual parameter dot plot, in which pulse signal (signal high; FSC-H; y-axis) versus signal area (FSC-A; x-axis) was displayed. To test the effect of different compounds (see Table I for details), cells were incubated overnight at 37°C and under 5% CO₂ with each respective compound, which was also present during data acquisition. When a mixture of compounds was used, they were also tested individually in the same batch of sperm. All compounds stock solutions were prepared in 10 mM HEPES.

**Subcloning of human and mouse Slo3 channels**

Full-length hSlo3 cDNA (catalog number MHS1010-7295475) was bought from Open Biosystems (Thermo Fisher Scientific, Pittsburg, PA, USA), cloned in pOX vector and then subcloned into pcDNA3.1(−) vector with standard ligation techniques using Nhe I and Hind III sites. A Hind III site was inserted into the hSlo3 cDNA sequence using oligonucleotide primers 5′ GGG AAG CTT GAG TCT AGA ACT AGT ATA GTG GCT 3′ and 5′ CGC ATT TAA CCC TCA CTA AAG 3′ synthesized by IBT/UNAM (Cuernavaca, Mexico). PCR was performed using high-fidelity Phusion™ DNA polymerase (New England BioLabs, Ipswich, MA, USA) starting with a denaturation step at 98°C for 30 s, followed by 30 cycles under the following conditions: 98°C for 10 s, 45°C for 20 s and 72°C for 70 s. The final elongation step was performed at 72°C for 10 min. The presence of a single product (~3500 bp) was confirmed in a 1% agarose gel and it was purified using a rapid gel extraction protocol by column (Fermentas). The amplified fragment was digested with appropriate restriction enzymes and ligated into the pcDNA3.1(−) vector using standard protocols. Recombinant clones were subsequently sequenced on both strands to confirm the identity of the construct. Mouse Slo3 channel cDNA (Santi et al., 2009) was used as a positive control and was directly subcloned into pcDNA3.1(−) vector digesting the clone with the Nhe I and Xho I restriction sites.

**Cell culture**

CHO cells were maintained in culture using Advanced Dulbecco’s modified Eagle’s medium (Gibco, Invitrogen) supplemented with 1% antibiotics and 10% BSA (Gibco, Life Technologies, Paisley, UK). Cells were grown in
plastic Petri dishes incubated in a humidity-controlled incubator at 37°C under 5% CO₂ (VWR Scientific 2100).

**Transient expression of hSlo3 channels in CHO cells**

Fifty percent of confluent CHO cells were transfected with 1 μg of the hSlo3 construct mixed with Lipofectamine (Invitrogen) according to the manufacturer's protocol. Transfected CHO cells were maintained in advanced DMEM medium and were used in the studies described >48 h post-transfection. Control experiments include cells transfected with empty pcDNA3.1(−) vector.

**Design and synthesis of anti-hSlo3 antibodies**

Two chicken anti-Slo3 channel antibodies (U1493 and U1504) were commissioned from New England Peptides using the antigen peptide Ac-CELKNSPHHFI-amide, corresponding to either amino acid residues 864–875 of human (NCBI Gene ID: 157855) or 869–880 of mouse (NCBI Gene ID: 16532) Slo3 channels.

**SDS-PAGE and western blot analysis**

Human sperm after swim-up separation (~3 × 10^6 cells/lane) were washed once (600g/5 min in phosphate buffered saline) at room temperature, resuspended and incubated in solubilization buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA; 1% v/v sodium deoxycholate, 1% v/v NP-40 and protease inhibitors complete (Roche, Mannheim, Germany) for 30 min at 4°C. Samples were then centrifuged at 16 000 g for 20 min, the supernatants were collected and 20 μg of total protein was mixed with loading buffer (500 mM Tris, 8 mM EDTA; 1 μM bromophenol blue/mL, 10% v/v SDS, 50% v/v glycerol and 5% v/v 2-mercaptoethanol). Samples were incubated at 70°C for 20 min, and then subjected to 7.5% SDS-PAGE. Proteins were electro-transferred (0.5 A/60 min, with a buffer solution, 190 mM glycine, 25 mM Tris, pH 8.6) to Immobilon P (Millipore) membranes (pre-treated according to the manufacturer’s instructions) in a semi-dry transfer cell (Bio-Rad). After blocking with 5% w/v fat-free milk, blots were incubated with anti-Slo3 (U1493) (1:5000), pre-immune serum (1:5000) or a mixture of anti-Slo3 and antigenic peptide (1:1 weight ratio) (New England Peptide LLC, Gardner, MA, USA) and then with the goat anti-chicken secondary antibody (1:1000) conjugated to horseradish peroxidase (Zymed, San Francisco, CA, USA) and developed with the chemiluminescent ECL kit (Thermo Fisher Scientific, Rockford, IL, USA).

### Immunodetection of hSlo3 channels in CHO cells

CHO cells transiently expressing hSlo3 channels were used to determine the specificity of the primary antibodies U1493 and U1504 (New England peptides). Aliquots of CHO cells were fixed with 4% v/v paraformaldehyde, permeabilized with 0.1% v/v Triton X-100 and incubated overnight at 4°C with primary antibody (only results with U1493 are shown) at 1:1000 dilution. Lastly, samples were incubated for 1 h with goat anti-chicken Alexa Fluor 488- or Alexa Fluor 594-conjugated antibodies (diluted 1:100, Molecular Probes-Invitrogen). Images were acquired in a confocal microscope Zeiss LSM510 META and analyzed using Zeiss LSM Image examiner software 4.2 (Carl Zeiss). Supplemenary immunodetection images were acquired in a confocal microscope FLUOVIEW FV1000 (Olympus) and analyzed using ImageJ 1.48 software.

### Membrane resting potential (Emᵣ) determinations in CHO cells

After transfection, 1 × 10⁶ cells were resuspended in modified Whittens buffer (100 mM N-methyl-D-glucamine, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 5.3 mM sucrose, 20 mM HEPES; pH 7.4) stained with 1 μM bis-(1,3-diethylthiobarbituric acid) trimethine oxonol (DiSBAC₂(3)) (Sigma-Aldrich) using a published procedure (Wolff et al., 2003) and incubated for 5–7 min at room temperature in 1 mL of modified Whittens buffer and increasing concentrations of KCl (6–66 mM). The basal fluorescence was recorded for 7 min using an Ocean Optics USB4000 spectrofluorometer operated by Spectra Suite (Ocean Optics, Inc., USA) with magnetic stirrer control (SIM Aminco), and coupled to a Cyan light-emitting diode (LED, Luxeon Star LXHL-LB3C, from LUMILEDS) and a 475–525 nm band-pass filter (Chroma Technology Corp.) for DiSBAC₂(3) excitation (ANASPEC). The LED was controlled by a custom-built power supply (700 mA). Emission light was obtained by setting the emission wavelength (λₑᵣ) from 550 up to 600 nm on the spectrofluorometer’s monochromator. Calibration was performed by adding 1 μL of gramicidin D (1 mM stock solution in DMSO; Sigma-Aldrich) and sequential additions of known concentrations of KCl to obtain final concentrations of 6.7, 10.7, 18.7, 30.7, 46.7 and 66.7 mM, corresponding to plasma Emᵣ (theoretical) of −80, −64, −49, −36, −25 and −3 mV, respectively. The fluorescence signal was further recorded for 2 min after each K⁺ addition, and the end values were used to calculate the membrane potential. These values were obtained using the Nernst equation, assuming an [K⁺] of 120 mM, according to Chávez et al. (2013). The final cell membrane potential was obtained by linearly interpolating the theoretical Emᵣ values versus arbitrary units of fluorescence from each trace. For pharmacological assays, the Emᵣ of Slo3-transfected

### Table 1  K⁺ and Na⁺ channel blockers.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Target</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba²⁺</td>
<td>1 mM</td>
<td>K⁺ channels</td>
<td>Von Beckerath et al. (1996); Leung et al. (2000); Martinez-López et al. (2009)</td>
</tr>
<tr>
<td>Clofilium</td>
<td>50 μM</td>
<td>Slo3 channels</td>
<td>Navarro et al. (2007); Zeng et al. (2011)</td>
</tr>
<tr>
<td>TEA⁺</td>
<td>10 mM</td>
<td>Slo1 + Slo3 (partial) channels</td>
<td>Tang et al. (2010)</td>
</tr>
<tr>
<td>TEA⁺</td>
<td>60 mM</td>
<td>Slo1 + Slo3 channels</td>
<td>Martinez-López et al. (2009); Tang et al. (2010)</td>
</tr>
<tr>
<td>IbTX</td>
<td>100 nM</td>
<td>Slo1 channels</td>
<td>Tang et al. (2010)</td>
</tr>
<tr>
<td>CbTX</td>
<td>100 nM</td>
<td>Slo1 channels</td>
<td>Tang et al. (2010)</td>
</tr>
<tr>
<td>SloTX</td>
<td>100 nM</td>
<td>Slo1 channels</td>
<td>Reviewed in Garcia-valdes et al. (2001)</td>
</tr>
<tr>
<td>TTX</td>
<td>60 nM</td>
<td>Na⁺ channels</td>
<td>Reviewed in Narahashi (2008)</td>
</tr>
</tbody>
</table>
CHO cells was measured in the presence of different K\textsuperscript{+} channel inhibitors (n = 6).

**Statistical analysis of data**

Cytometry data were analyzed automatically using FACS Diva and FlowJo software. After gating to exclude debris and dead cells (~12,000–15,000 cells were left for analysis (the average number of cells per condition/experiment is shown in Supplementary data, Tables S1 and SII). We present the cytometry data as histograms of percentage of the maximum (% Max) versus fluorescence, obtained by normalizing to the peak height at the mode of the distribution—so the maximum y-axis value in the absolute count histogram becomes 100% of total. The histograms, mean (arithmetic mean), standard deviation (SD) and geometric mean (GM) were automatically calculated by FlowJo Software. The average mean and the average GM per experimental condition and their corresponding SD were calculated according to supplementary equations and presented in Supplementary data, Tables SI and SII. All these parameters were compared with their corresponding control and analyzed using a t-test.

The percentage of inhibition was calculated as follows: cytometry data allows us to determine directly the number of cells with a given fluorescence. To consider that a cell was hyperpolarized we established a fluorescence intensity threshold, or gate, which was defined as the fluorescence intensity value reached at the point where the log distribution of non-capacitated cells (considered as non-hyperpolarized) included up to 95% of the total number of events counted in this condition (95th centile). A cell with a fluorescence intensity value equal to or higher than the fluorescence intensity gate was considered as hyperpolarized. We then compared the number of cells that were hyperpolarized in the presence of an inhibitor (in the same sperm batch) and calculated the % of inhibition according to the equation presented in Supplementary data, Tables SIII and SIV. These tables show the average number of hyperpolarized cells, the average of total cells counted, the average of percentage of inhibition, the SD and the standard error of the mean (SEM) of the percentage of inhibition.

Statistical analysis was performed using the R 2.11.1 program (The R foundation for statistical computing, 2010). Analysis of variance (ANOVA) and Tukey’s test for multiple comparisons were used to compare either the parameters of relative fluorescence intensity in capacitated versus non-capacitated sperm samples using the Em-sensitive dye DiSC\textsubscript{3}-(5). Figure 1 shows a representative flow cytometry experiment. A plot of cell complexity (SSC-A) versus cell size (FSC-A) is shown in A (Fig. 1A). The population was gated to eliminate cell debris and aggregates (Fig. 1B); the selection of individual cells was confirmed using the plot of FSC-H versus FSC-A that should give a straight line (Fig. 1C). In order to analyze selectively only the Em of living cells, we always co-stained sperm with PI (5 min prior to acquiring the data); normally we obtained a cellular viability >90%. In non-capacitating conditions, we observed that the fluorescence intensities in the sperm population showed a log-normal distribution with a range of relative fluorescence intensity from 5,000 up to 40,000. In capacitating conditions, a subpopulation of live capacitated cells exhibited a higher fluorescence and hyperpolarized variables (Fig. 1D−F). In this case the range of relative fluorescence intensity was from 3,000 up to 60,000. The mean and the GM for both non-capacitated and capacitated conditions are shown in Supplementary data, Table S1, the difference between them was statistically significant (P = 0.001). However, we found a large heterogeneity in the percentage of hyperpolarized sperm among samples from different individuals. Despite this large heterogeneity, in 16 out of 18 experiments a subpopulation of hyperpolarized cells in spermatozoa exposed to capacitating conditions was observed. Figure 2 shows three representative examples of a small (Fig. 2A), a medium (Fig. 2B) or a large hyperpolarized subpopulation during capacitation (Fig. 2C). Correlation analysis of high [Ca\textsuperscript{2+}] and pH, sperm subgroups versus the percentage of cells in hyperpolarized subpopulations indicated a strong correlation between high [Ca\textsuperscript{2+}] and hyperpolarization variables in capacitated sperm (ρ = 0.96), whereas a non-linear Spearman’s correlation was observed for high pH and hyperpolarized variables (ρ = 0.95) (Supplementary data, Fig. S3).

**Slo3 channels are expressed in human sperm**

Membrane proteins from human sperm were separated by SDS-PAGE and probed with an antibody against Slo3. A band of the predicted molecular mass of 130 kiloDaltons (kDa) was observed as well as band with lower molecular weight (probably due to protein degradation). Both bands were not detected when the sample was incubated with the pre-immune serum or with the mixture of the antibody and the antigenic peptide (Fig. 3).

**Results**

A subpopulation of human sperm hyperpolarizes under capacitating conditions

We set out to determine whether it would be possible to identify a subpopulation of hyperpolarized human sperm using flow cytometry—a technique that provides information for a large population of cells, but with single-cell resolution. As a proof of concept we first demonstrated that we were able to detect pH\textsubscript{i}, [Ca\textsuperscript{2+}], and Em changes, by artificially increasing these parameters using NH\textsubscript{4}Cl (20 mM), ionomycin (20 μM) or valinomycin (1 μM), respectively. These results are shown in Supplementary data, Fig. S1. Then we detected the increase of pH\textsubscript{i} and [Ca\textsuperscript{2+}], known to occur during capacitation using this technique (Supplementary data, Fig. S2). We were indeed able to detect the expected shift to the right in fluorescence values, indicating that both parameters increased in a subpopulation of human sperm upon incubation under capacitating conditions. Next, we proceeded to explore whether it was possible to identify a hyperpolarized subpopulation of cells in capacitated versus non-capacitated sperm samples using the Em-sensitive dye DiSC\textsubscript{3}(5). Figure 1 shows a representative flow cytometry experiment. A plot of cell complexity (SSC-A) versus cell size (FSC-A) is shown in A (Fig. 1A). The population was gated to eliminate cell debris and aggregates (Fig. 1B); the selection of individual cells was confirmed using the plot of FSC-H versus FSC-A that should give a straight line (Fig. 1C). In order to analyze selectively only the Em of living cells, we always co-stained sperm with PI (5 min prior to acquiring the data); normally we obtained a cellular viability >90%. In non-capacitating conditions, we observed that the fluorescence intensities in the sperm population showed a log-normal distribution with a range of relative fluorescence intensity from 5,000 up to 40,000. In capacitating conditions, a subpopulation of live capacitated cells exhibited a higher fluorescence and hyperpolarized variables (Fig. 1D−F). In this case the range of relative fluorescence intensity was from 3,000 up to 60,000. The mean and the GM for both non-capacitated and capacitated conditions are shown in Supplementary data, Table S1, the difference between them was statistically significant (P = 0.001). However, we found a large heterogeneity in the percentage of hyperpolarized sperm among samples from different individuals. Despite this large heterogeneity, in 16 out of 18 experiments a subpopulation of hyperpolarized cells in spermatozoa exposed to capacitating conditions was observed. Figure 2 shows three representative examples of a small (Fig. 2A), a medium (Fig. 2B) or a large hyperpolarized subpopulation during capacitation (Fig. 2C). Correlation analysis of high [Ca\textsuperscript{2+}] and pH, sperm subgroups versus the percentage of cells in hyperpolarized subpopulations indicated a strong correlation between high [Ca\textsuperscript{2+}] and hyperpolarization variables in capacitated sperm (ρ = 0.96), whereas a non-linear Spearman’s correlation was observed for high pH and hyperpolarized variables (ρ = 0.95) (Supplementary data, Fig. S3).

**Human sperm hyperpolarization depends on a K\textsuperscript{+} permeability increase sensitive to Slo1 and Slo3 K\textsuperscript{+} channels inhibitors**

To characterize the role of K\textsuperscript{+} flux and the channel(s) involved in human sperm hyperpolarization, we incubated the cells in the presence of high K\textsuperscript{+} (60 mM) and in the presence of several K\textsuperscript{+} channel antagonists. Figure 4 shows representative results indicating that high [K\textsuperscript{+}], completely inhibited the hyperpolarization observed in sperm samples in all cases (96.6 ± 1.7%; n = 11), strongly suggesting that this process depends on a K\textsuperscript{+} permeability. This hyperpolarization was partially sensitive to Ba\textsuperscript{2+} (1 mM), TEA\textsuperscript{+} (60 mM) and clofilium (50 μM) with 74.1 ± 10.5, 78.0 ± 12 and 74.2 ± 12.5% of blockade, respectively. In order to explore the possible participation of other K\textsuperscript{+} channels, capacitated sperm were incubated with TEA\textsuperscript{+} (10 mM), charybotoxin...
Hyperpolarization was inhibited by 60.4 ± 10.4, 69.5 ± 8.9, 52.6 ± 14.7 and 55.5 ± 9.2% with TEA +, CbTX, SloTX and IbTX, respectively. At 10 mM, TEA + acts as a general K+ channel inhibitor but only partially inhibits Slo3 channel currents; CbTX, SloTX and IbTX are more specific for Slo1, suggesting that at least two different K+ channels (probably Slo1) participate in the capacitation-associated hyperpolarization in human sperm. Consistently, sperm hyperpolarization was insensitive to TTX (60 nM), a voltage-dependent Na+ channel blocker, with a negligible percentage of blockade. Intriguing results were obtained when combining the blockers used above. The presence of clofilium with either IbTX, SloTX or TEA (10 mM) decreased the % of inhibition of the hyperpolarization compared with each inhibitor alone. In contrast, combining CbTX with either clofilium or IbTX produced the same inhibition as CbTX alone. The general, inhibition exerted by the blockers was consistent among experiments, although we did observe some variation in the extent of blockage between samples. All comparisons were made within the same batches of sperm. The summary of the percentage of inhibition is presented in Figs 4B and 5B and flow cytometry statistical data are presented in Supplementary data, Tables SI–SIV.

Heterologous expression and pharmacological profile of hSlo3 channels in CHO cells

As an alternative strategy to characterize hSlo3 channels, we expressed them in CHO cells in order to evaluate their pharmacological profile (only mSlo3 profile has been reported) and attempt to correlate it with the inhibitory profile of the capacitation-induced hyperpolarization observed in our flow cytometry studies. First, we verified the expression of the channel in CHO cells using an anti-Slo3 antibody. Control non-transfected CHO cells showed no signal in the presence of anti-Slo3 antibody, ruling out any endogenous epitopes for this antibody (Fig. 6A, control primary Ab). In contrast, Slo3 channels were clearly detected in the plasma membrane of hSlo3- and mSlo3-transfected cells (Fig. 6A, hSlo3 or mSlo3 primary Ab). However, most of the Slo3 signal was intracellular, probably due to its retention in an unidentified trafficking pathway organelle (Supplementary data, Fig. S4), precluding the electrophysiological recording of Slo3 macroscopic ion currents likely due to the absence of Slo3 ancillary subunits (Yang et al., 2011).

On the other hand, hSlo3-transfected cells showed no signal in the presence of anti-Slo3 antibody, ruling out any endogenous epitopes for this antibody (Fig. 6A, control primary Ab). In contrast, Slo3 channels were clearly detected in the plasma membrane of hSlo3- and mSlo3-transfected cells (Fig. 6A, hSlo3 or mSlo3 primary Ab). However, most of the Slo3 signal was intracellular, probably due to its retention in an unidentified trafficking pathway organelle (Supplementary data, Fig. S4), precluding the electrophysiological recording of Slo3 macroscopic ion currents likely due to the absence of Slo3 ancillary subunits (Yang et al., 2011).

The presence of Slo3 in human sperm is confirmed by immunoblotting. Immunoblotting experiments using protein extracts from human sperm cells. (A) The left lane (Ab) shows the signal with the anti-Slo3 antibody (U1493), the middle lane corresponds to samples incubated with the serum before the immunization (Preimmune) and the right lane shows the signal of the anti-Slo3 antibody pre-incubated with the antigenic peptide (Ab + Pep). (B) The Immobilon P membrane was stained with amido black to show that similar amounts of protein were transferred to the membrane in each lane. The molecular weight markers in kiloDaltons (kDa) are shown to the left of each panel. The results presented are representative of at least three independent experiments from three different semen donors.
Figure 4 The capacitation-associated hyperpolarization of human sperm is inhibited by different K^+ channel blockers. (A) Histograms of percentage of the maximum (% Max) versus DiSC3(5) fluorescence for non-capacitated (red) and capacitated (blue) sperm incubated with different blockers: KCl 60 mM (black), Ba^{2+} 1 mM (light green), clofilium 50 μM (aqua blue), TEA 60 mM (purple), TEA 10 mM (pink), IbTX 100 nM (orange), CbTX 100 nM (green), SloTX 100 nM (light pink), and TTX 60 nM (gray), an Na^+ Channel inhibitor, a negative control. The shift to left in the fluorescence traces in the presence of inhibitor indicates that the hyperpolarization was inhibited, the percentage of inhibition is presented in (B). The bars represent the mean ± SD of n = 5–16. In all cases, P < 0.01.

Figure 5 Combining two K^+ channel blockers did not produce an additive inhibition of the capacitation-induced membrane hyperpolarization. (A) Histograms of percentage of the maximum (% Max) versus DiSC3(5) fluorescence for non-capacitated (red) and capacitated (blue) sperm incubated with different combinations of K^+ channel blockers: clofilium 50 μM + IbTX 100 nM (orange), clofilium 50 μM + CbTX 100 nM (green), clofilium 50 μM + SloTX 100 nM (light pink), clofilium 50 μM + TEA 10 mM (pink), and IbTX 100 nM + CbTX 100 nM (brown). The shift to left in the fluorescence traces in the presence of inhibitor indicates that the hyperpolarization was inhibited, the percentage of inhibition is presented in (B). Bars represent the mean ± SD of n = 5–6. In all cases, P < 0.01.
Mock-transfected cells, using an empty vector, also showed a depolarized Em (−26 ± 9 mV) close to that of native CHO cells, ruling out a non-specific effect on membrane potential solely due to vector transfection. In contrast, CHO cells transfected with hSlo3 or mSlo3 channels exhibited a hyperpolarized Em of −59 ± 4 and −62 ± 4 mV, respectively, strongly suggesting the functional expression of Slo3 channels. The hyperpolarized Em in transfected CHO cells was very sensitive to high \([K^+]_o (60 \text{ mM}), \text{Ba}^{2+} (1 \text{ mM}), \text{TEA}^+ (60 \text{ mM})\), and clofilium \((50 \mu\text{M})\) with Em of −3 ± 0.3, −25 ± 12, −11 ± 5 and 9 ± 3 mV, respectively, whereas the hyperpolarized Em in CHO cells was partially inhibited by 10 mM \text{TEA}^+ (−46 ± 6 mV) (Fig. 6C), consistent with the pharmacological profile reported for mSlo3 (Martínez-López et al., 2009; Tang, et al., 2010).

Altogether our data suggest the participation of hSlo3 and Slo1 \(K^+\) channels in the hyperpolarization observed in capacitated human sperm, but due to the differential sensitive and non-strict specificity of the blockers used, the participation of other \(K^+\) channels cannot be ruled out.

**Discussion**

Various findings suggest that hyperpolarization is essential for sperm to acquire the ability to undergo a physiological AR. These observations lead to the proposal that an Em hyperpolarization is necessary and sufficient for the AR to take place (De La Vega-Beltran et al., 2012). Despite the importance of this event in fertilization of several species, human sperm membrane hyperpolarization during capacitation has not been established. Several studies have measured human sperm membrane potential but have not compared in detail non-capacitated versus capacitated cells. For example, Linares-Hernandez et al. (1998) reported that...
membrane potential of non-capacitated human spermatozoa (no incubation after sperm separation from semen) lays around $-40 \pm 16 \text{ mV}$ and Patrat et al. (2002) reported a value of $-58 \pm 2 \text{ mV}$ after capacitation. Considering only the values from these papers one can infer that a hyperpolarization took place during capacitation; however, their experimental conditions such as time of incubation and composition of solutions were quite different and the values cannot be compared directly. In all cases the $E_{m}$ for non-capacitated sperm was measured immediately after the separation of sperm, instead of what is normally done at least for mouse sperm, where sperm cells are incubated for the same amount of time in media that either support or do not support capacitation (absence of BSA). Interestingly, there is a study by Calzada and Tellez (1997) aimed to compare the $E_{m}$ of sperm cells from fertile versus non-fertile individuals in solutions with different ionic compositions. They found that regardless of the ionic composition of the media, sperm from fertile men always display a more hyperpolarized $E_{m}$ ($-75$ to $-35 \text{ mV}$) compared with sperm from infertile men ($-35$ to $-10 \text{ mV}$), suggesting that $E_{m}$ plays an important role in sperm fertility.

Prior to the present report, there is only one flow cytometry study, in which Brewis et al. (2000) reported that capacitation in human sperm is associated with a hyperpolarization. However, this study did not determine the percentage of hyperpolarized cells observed after sperm capacitation neither the statistical significance of the results nor the identity of the molecular entities involved in the hyperpolarization. In our study we confirmed that using flow cytometry, it is possible to clearly distinguish a subpopulation of hyperpolarized human sperm after capacitation. We explored a significant number of donors (19 individuals) and observed that in average $57 \pm 23\%$ of the sperm population shifted to a more hyperpolarized $E_{m}$ under capacitating conditions. However, the percentage of hyperpolarized cells among different donors is highly variable (see Fig. 2). This large variability may explain why the hyperpolarization associated with capacitation has not been detected in human sperm using conventional fluorometry, in which only average values are obtained that may mask the difference among subpopulations. The presence of hyperpolarized sperm subpopulations has also been recorded in mouse sperm. Arnoult et al. (1999) measured $E_{m}$ in individual mouse spermatozoa using di8-ANEPPS, a fast voltage sensitive dye and documented that capacitated mouse sperm consists of at least two groups: one hyperpolarized ($-80 \text{ mV}$) possibly representing capacitated spermatozoa and another of non-capacitated cells with a resting $E_{m}$ $\sim -43 \text{ mV}$.

Previous work from several laboratories including ours have proposed the involvement of epithelial Na$^{+}$ channels, CFTR, K$^{+}$ channels and other Cl$^{-}$ transporters during mouse sperm membrane hyperpolarization. In spite of this, Chávez et al. (2013) recently reported that although mouse sperm $E_{m}$ before capacitation is governed by K$^{+}$, Cl$^{-}$ and Na$^{+}$ permeabilities, the hyperpolarization observed during this process is mainly due to the pH activation of Slo3 channels. It is becoming apparent that there are differences of quantity and biophysical characteristics of the ion channels present in diverse mammalian sperm species possibly tailoring their function to female genital tract characteristics. At the beginning of this study, the presence of Slo1 or Slo3 had not been documented in human sperm, although the latter has now been heterologously expressed and electrophysiologically recorded in oocytes by Leonetti et al. (2012). Very recently Mansell et al. (2014) reported the presence of at least three distinct cation channels in human sperm, two of which may conduct K$^{+}$ (possibly Slo1 and Slo3). We detected by western blot analysis the presence of Slo3 channels in human sperm and tested different K$^{+}$ channel blockers to explore if Slo3 and/or Slo1 K$^{+}$ channels would have a preponderant role in the hyperpolarization of human sperm. We found that human sperm $E_{m}$, hyperpolarization was completely inhibited by high [K$^{+}$]o (60 mM) strongly suggesting the participation of K$^{+}$ fluxes in this process. This hyperpolarization displayed a differential sensitivity to various K$^{+}$ channel blockers; Ba$^{2+}$ (1 mM), TEA$^{+}$ (60 mM) and clofilium (50 μM) strongly inhibited indicating the participation of Slo3 K$^{+}$ channels. Previous reports indicate that Ba$^{2+}$ at this concentration acts as a general K$^{+}$ channel inhibitor, whereas the last two compounds are more specific Slo3 K$^{+}$ channel blockers (reviewed in Navarro et al., 2007; Tang et al., 2010). TEA$^{+}$ (10 mM), charybdotoxin (CbTX, 100 nM), slotoxin (SloTX, 100 nM) and ibetoxin (IbTX, 100 nM) inhibited to a similar extent the hyperpolarization, suggesting the contribution of Slo1 channels.

The hyperpolarization was insensitive to tetrodotoxin (TTX, 60 nM) ruling out the participation of voltage-dependent Na$^{+}$ channels. The development of Slo K$^{+}$ channel inhibitors of greater specificity can help provide more precise information about the contribution of Slo3 and/or Slo1 K$^{+}$ channels in human sperm membrane hyperpolarization. Altogether our results suggest the presence of different K$^{+}$ channels within the human sperm membrane, which could contribute to determine $E_{m}$ and/or participate in the hyperpolarization observed during capacitation. Interestingly, in mouse sperm the capacitation-induced hyperpolarization mainly depends on Slo3 channels. Our pharmacological results suggest that in human sperm different K$^{+}$ channels may be necessary to achieve the hyperpolarization of the membrane potential during capacitation. This is consistent with the recent report by Mansell et al. (2014), which suggests that the channel repertoire of human sperm is more complex as previously envisioned. For example, Mannowitz et al. (2013) reported that the main K$^{+}$ channel that can be recorded electrophysiologically in human sperm is Slo1, which is located at the flagella. In this work they show that the K$^{+}$ current recorded in non-capacitated human sperm is regulated by Ca$^{2+}$ but not by pH. The current is almost completely inhibited ($\sim 80\%$ of inhibition) by two different Slo1 channel inhibitors, namely CbTX (1 μM) and IbTX (100 nM); whereas paxilline (100 nM), a third Slo1 inhibitor, blocked only 60% of the sperm K$^{+}$ current. In the present report, CbTX (100 nM), SloTX (100 nM) and IbTX (100 nM) partially inhibited the capacitation-associated hyperpolarization (between 55 and 70%), consistent with the presence of Slo1 K$^{+}$ channels in human sperm. However, incubation of capacitated human sperm with clofilium (50 μM), a more specific Slo3 blocker also inhibited the capacitation-associated hyperpolarization by $\sim 65\%$, suggesting that Slo3 channels are also relevant in this maturational process. Indeed, Slo3 channels are expressed in human sperm as determined by western blot analysis using swim-up isolated sperm to avoid contamination from other cell types (Fig. 3). Although we tried to confirm these results by doing immunocytochemistry, our antibodies did not yield reliable signals. Recently, human sperm Slo3 channels were heterologously expressed in Xenopus oocytes (Leonetti et al., 2012) where they were found to be activated by intracellular alkalization. Unfortunately, the authors did not perform a pharmacological profile. Expressing hSlo3 channels in CHO cells we indirectly demonstrated their sensitivity to clofilium (50 μM), as it was shown for Slo3 currents in mouse sperm (Zeng et al., 2011). In contrast, a previous report showed that Slo1 channels are insensitive to clofilium (100 μM) (Fernández-Fernández et al., 2002). Consistently
Conflict of interest

None declared.

References


Arnot C, Cardullo RA, Lemos JR, Florman HM. Activation of mouse sperm T-type Ca2+ channels by adhesion to the egg zona pellucida. Proc Natl Acad Sci USA 1996;93:13004—13009.


Garcia-valdes J, Zamudio FZ, Toro L, Possani LD. Slotoxin, K KTx1.11, a new peptide blocker of MaxiK channels that differentiates between K and K + L (L 1 or L 4) complexes. FEBS Lett 2001;505:369—373.

Hernández-González EO, Sosnik J, Edwards J, Acevedo JJ, Mendoza-Lujambio I, López-González I, Visconti PE. Sodium and epithelial sodium channel expression in these cells and the potential opening of endogenous swelling-activated Cl− currents (Gabriel et al., 1992; Skryma et al., 1994; Li et al., 2000; Punke et al., 2003; Gamper et al., 2005).

The pharmacological profile observed for the capacitation-associated hyperpolarization in human sperm could be explained by at least by two hypotheses: (i) Slo1 and Slo3 heterotetramers participate in the signaling cascade involved in the capacitation-induced hyperpolarization explaining why it is inhibited to the same extent by Slo1 or Slo3 channel antagonists or (ii) Slo1 and Slo3 channels form heterodimers resulting in a functional channel with a distinct pharmacological profile. Our results combining blockers did not cause an additive inhibition, instead in most cases it caused a smaller effect than the individual blockers, except for the CbTx/IbTx and clomidou/CbTX combinations. These results were very reproducible (n = 5), although difficult to understand. It is possible that if the human sperm K+ channels are formed by heterotetramers of different Slo subunits, the interaction of both toxins/blockers may be sterically hindered in such a way that neither of them may exert their full action. In the other cases, if the blockers target the same channel, the resultant inhibition is the same as when just one of the blockers is present. Though, we favor the hypothesis of the presence of Slo heterotetramers in human sperm, both proposals deserve further investigation.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

Acknowledgements

The authors thank Andrés Saralegui, José Luis De la Vega, Xóchitl Alvarado, Yolectl Sánchez, Shirley Ainsworth, Elizabeth Mata, Erika Melchy, Graciela Cabeza and Marcela Ramírez for technical assistance. We thank Juan Manuel Hurtado, Roberto Rodríguez, and Arturo Ocádiz for computer services.

Authors’ roles

I.L.G., P.T.R., O.S.C. and A.S.L. contributed substantially to the acquisition and analysis of data; drafting the article and final approval of the version to be published. I.L.G., A.D., C.M.S. and C.L.T. substantially contributed to the study design, revising critically the article for important intellectual content and final approval of the version to be published. C.L.T. managed the project.

Funding

This work was supported by National Institute of Health (NIH) Grants R01 HD038082-07A1 and RO1 HD069631-04 (to Pablo Visconti subcontract to A.D. and to C.S., respectively), Consejo Nacional de Ciencia y Tecnología (CONACyT-Mexico) (99333 to C.T., 84362 to I.L.G. and 128566 to A.D. and C.T.; Dirección General de Asuntos del Personal Académico/Universidad Nacional Autónoma de México (DGAPA/UNAM) (IN25406 to A.D., IN217210: IN204914 to I.L.G. and IN202212-3 to C.T.); The Alexander von Humboldt Foundation (to C.T.).

with previous reports in the same ionic conditions, we found that Em, of CHO cells was depolarized, probably due to low endogenous K+ channel expression in these cells and the potential opening of endogenous swelling-activated Cl− currents (Gabriel et al., 1992; Skryma et al., 1994; Li et al., 2000; Punke et al., 2003; Gamper et al., 2005).