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Human sperm capacitation is necessary for SNARE assembly in neurotoxin-resistant complexes

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ABSTRACT

Background: Capacitation is not a well-defined process, required for the acrosome reaction triggered by physiological stimuli. In vitro, capacitation is achieved by sperm incubation in artificial media supplemented with HCO_3^-, Ca^{2+}, and albumin. The role of capacitation in the membrane fusion machinery required for acrosomal exocytosis is not well-known. SNARE proteins are fundamental for intracellular membrane fusion and acrosomal exocytosis. We have previously shown that in capacitated spermatozoa, the fusion machinery is maintained in an inactive state until the acrosome reaction is initiated. In particular, SNARE proteins are assembled in neurotoxin-resistant complexes.

Objective: This work aimed to study the dynamic changes of SNARE complexes during capacitation.

Materials and Methods: The light chain of tetanus and botulinum neurotoxin has been widely used to study the configuration of SNARE proteins. For this purpose, we developed a recombinant light chain of tetanus neurotoxin linked to a polyarginine peptide. This membrane-permeant protein was able to cleave cytosolic VAMP2 (a SNARE protein required for acrosome reaction) when present in a monomeric configuration.

Results: The results show that the VAMP2 is cleaved by the membrane-permeant tetanus neurotoxin in non-capacitated spermatozoa, indicating that, before capacitation, SNAREs are not assembled in stable toxin-resistant complexes. However, 2 h of incubation in a capacitation medium containing albumin was sufficient to render VAMP2 insensitive to the toxin.

Discussion: We conclude that during capacitation, the SNARE proteins become engaged in stable fully assembled *cis*-SNARE complexes. This step is likely essential to prevent untimely activation of the membrane fusion machinery.

Conclusion: We propose that capacitation promotes the stabilization of the membrane fusion machinery required for acrosomal exocytosis in preparation for the stimulus-triggered acrosome reaction.

INTRODUCTION

After ejaculation, spermatozoa must undergo capacitation to be able to fertilize the oocyte (Austin, 1951; Chang, 1951). This process is achieved by the sperm contact with the female reproductive tract environment and is characterized by a special motility pattern and the ability to carry out the acrosome reaction under physiological stimuli (Visconti, 2009). Many changes have been described during capacitation, including plasma membrane remodeling and flagellum hyperactivation. At the plasma membrane, capacitation promotes a membrane fluidity increment and the efflux and mobilization of cholesterol to the head-apical zone (Salicioni *et al.*, 2007). In vitro, the capacitation can be mimicked by incubation of the spermatozoa in medium supplemented with HCO_3^-, Ca^{2+}, and bovine serum albumin (BSA) (Chang, 1951; Bedoford, 1962). The presence of BSA is necessary to promote cholesterol efflux and membrane remodeling (Salicioni *et al.*, 2007).

The acrosome reaction is an absolute requirement for fertilization. Only reacted spermatozoa can fuse with the oocyte to start the development of an embryon (De Lamirande *et al.*, 1997; Stival *et al.*, 2016). The acrosome reaction is a regulated exocytosis event with special characteristics (Belmonte *et al.*, 2016). As is the case in many intracellular fusion events, the acrosome reaction requires the participation of SNAREs (Soluble NSF Attachment Protein Receptor), which mediate the opening of multiple fusion pores and result in the formation

of hybrid vesicles (De Blas *et al.*, 2005; Zanetti & Mayorga, 2009).

SNAREs are membrane-associated proteins that, when active and present in separate organelles, can form *trans*-complexes, bridging membranes toward fusion. This membrane proximity will eventually promote the mixing of the bilayers and the opening of fusion pores. When all the SNAREs are in the same membrane, they form an inactive *cis*-complex that needs to be disassembled by NSF (N-ethylmaleimide sensitive factor) and α-SNAP (α-soluble NSF attachment protein) that requires ATP hydrolysis to become active. Most exocytic events are triggered by cytosolic increases in calcium. Synaptotagmins are calcium-binding proteins that interact with the SNARE machinery, providing calcium sensitivity to the fusion process (Südhof & Rothman, 2009).

Our group has contributed several experimental evidence to the molecular processes involved in acrosomal exocytosis and the role of SNAREs in this unique membrane fusion event. These results are consistent with the following scenario: in capacitated spermatozoa before stimulation, the fusion machinery is inactive whereby SNAREs are assembled in *cis*-complexes (De Blas *et al.*, 2005) and NSF and synaptotagmin are in the inactive phosphorylated state (Roggero *et al.*, 2005; Zarelli *et al.*, 2009). Upon sperm-stimulation, a calcium influx from the extracellular medium promotes synaptotagmin and NSF dephosphorylation by the phosphatases calcineurin and PTP1B, respectively (Zarelli *et al.*, 2009; Castillo *et al.*, 2010). NSF and α-SNAP dissociate the *cis*-SNARE complexes (Michaut *et al.*, 2001; Tomes *et al.*, 2002; Zarelli *et al.*, 2009). Now, the active SNAREs in the plasma membrane and outer acrosomal membrane assemble in a *trans*-SNARE complex. The process stops there until a second calcium increase (coming from an efflux from the acrosomal granule) drives the final fusion step, which requires the trans-SNARE complexes, complexin, and synaptotagmin (De Blas *et al.*, 2005; Roggero *et al.*, 2005).

A powerful tool to study SNARE proteins are the light chains of botulinum (BoNT) and tetanus (TeNT) neurotoxins (Chen *et al.*, 2008). Particularly, monomeric VAMP2 (a SNARE present in the sperm membrane) is cleaved by TeNT and BoNT-B, whereas it is resistant to TeNT and cleaved by BoNT-B when engaged in loose *trans*-SNARE complexes. However, the protein is resistant to both TeNT and BoNT-B when assembled in tight *cis*-SNARE complexes (De Blas *et al.*, 2005) (De Blas *et al.*, 2005).

Many of our observations indicate that in resting capacitated spermatozoa, the fusion machinery is kept inactive in preparation for the stimuli that trigger exocytosis. Nevertheless, it is not known if this particular condition of the fusion mechanism is influenced by capacitation. Some years ago, Gadella's group reported that pig capacitated spermatozoa had *trans*-SNARE complexes assembled, which were absent in non-capacitated spermatozoa (Tsai *et al.*, 2010).

In the present work, we proposed to study how capacitation modulates SNARE complexes and primes them for acrosomal exocytosis in human spermatozoa. For this purpose, we developed a membrane-permeant tetanus toxin (TeNT-CPP), which permits evaluation of the SNARE complex assembly conditions in non-capacitated and capacitated spermatozoa. Our findings showed that in non-capacitated spermatozoa, VAMP2 is not protected in a neurotoxin-resistant complex, suggesting that the capacitation process strongly influences the dynamics of SNARE proteins in preparation for the acrosomal reaction.

MATERIALS AND METHODS

Reagents

Pisum sativum lectin labeled with fluorescein isothiocyanate (FITC-PSL) and bovine serum albumin (BSA) were from Sigma-Aldrich S.A. Ni-NTA-agarose was from Qiagen (Tecnolab SA, Buenos Aires, Argentina); DL-dithiothreitol (DTT) and isopropyl b-D1-thiogalactopyranoside (IPTG) were from ICN (Eurolab SA, Buenos Aires, Argentina). Mouse anti-phospho-tyrosine antibody (PY20, sc-508AC) was from Santa Cruz and anti-VAMP2/Syb2 (mouse monoclonal, clone 69.1, purified IgG) was from Synaptic Systems. Horseradish peroxidase (HRP)-coupled goat anti-mouse and Cy3-labeled goat anti-mouse were from Jackson ImnunoResearch (West Grove, PA, USA). N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) was purchased from Molecular Probes (Eugene, OR, USA). A23187 was from Alomone Labs (Alomone Labs., Jerusalem, Israel). All other chemical reagents were of analytical grade and were purchased from ICN or Sigma-Aldrich.

Tetanus toxin conjugation with cell-penetrating peptides

The plasmid DNA encoding His6-tetanus toxin light chain in pQE3—Qiagen—(previously provided by T. Binz from Medizinische Hochschule Hannover, Hannover, Germany) was transformed into *E. coli* XL-1Blue (Stratagene, La Jolla, CA, USA), and protein expression was induced overnight at 20 °C with 0.2 mM IPTG. Purification of recombinant His_6-tetanus toxin (TeNT) was accomplished according to the QIAexpressionist (www.qiagen.com). Conjugation between His_6-TeNT and the peptide RRRQRRKRRRQ (CPP) was performed using Trilinks Biotechnology Kits, according to the manufacturer's instruction. Briefly, the CPP was linked to succinimidyl-6-hydrazino-nicotinamide (S-HyNic), and His6-TeNT was linked to succinimidyl-4-formyl-benzamide (S-4FB), through primary amines. HyNic-modified peptide and 4FB-modified protein (3:1 molar equivalent) were cross-linked by incubating for 3 h at RT in the presence of aniline (100 mM) as a catalyst. The conjugate was further purified using a Sephadex G25 column after the conjugation reaction. Purity was confirmed by SDS-PAGE analysis.

Human sperm sample preparation and acrosome exocytosis

Human semen samples were obtained from normal, healthy donors. The informed consent and protocol for semen handling were approved by the Ethics Committee of the Medical School of the National University of Cuyo. Semen was allowed to liquefy for 30–60 min at 37 °C. We used a swim-up protocol to isolate highly motile spermatozoa in human tubal fluid media (as formulated by Irvine Scientific) supplemented with 0.5% BSA (capacitating conditions) or without BSA (not capacitating conditions) for 1 h at 37 °C in an atmosphere of 5% CO_2 /95% air. Sperm concentration was adjusted to 5–10 × 10^6 cells/mL before incubating for 3 h under capacitating conditions. For acrosome reaction assays, we added inhibitors and stimulants sequentially, as indicated in the figures. Spermatozoa were spotted on Teflon-printed slides, air dried, and fixed/permeabilized in ice-cold methanol for 1 min. Acrosomal status was evaluated by indirect or classic staining with 25 μg/mL of FITC-PSL, as previously described (Zoppino *et al.*, 2012), using a Nikon Optiphot II microscope (Nikon, Inc., Tokyo, Japan) equipped with epifluorescence optics. At least 200 cells were scored from each

condition of at least three independent experiments. Negative (no stimulation) and positive (10 μM A23187 – 7 μM progesterone) controls were included in all experiments. For each experiment, the data were normalized by subtracting the number of reacted spermatozoa from the negative control (range 5–10%) to the values of all experimental condition. The resulting values (after subtracting the negative control) of each experimental conditions were expressed as a percentage of the acrosome reaction observed in the positive control (A23187 range 30–50%; progesterone range 7–14%). After normalization, the negative and positive control of each experiment were 0% and 100%, respectively, and therefore, the values of negative and positive controls from all experiments do not include S.E.M. Differences between experimental and control conditions were tested by Parametric multiple comparison and Tukey–Kramer tests. Only significant differences ($p < 0.05$) are discussed in the text.

Indirect immunofluorescence

Sperm cells were spotted on poly-L-lysine-covered slides, fixed in 2% paraformaldehyde in PBS for 10 min at RT and incubated in 100 mM NH_4 Cl for 30 min at RT. The spermatozoa were permeabilized and blocked in PBS/0.2% BSA/0.05% saponin for 30 min at RT. Cells were labeled with mouse anti-VAMP2 antibody (20 μg/mL in PBS/0.2% BSA/0.05% saponin) overnight at 4 °C, followed by a Cy3-labeled anti-mouse secondary antibody (4 μg/mL in in PBS/0.2% BSA/0.05% saponin) for 1 h at RT. Slides were washed three times for 5 min with PBS between incubations. Slides were mounted in 1% propyl-gallate in PBS/50% glycerol. Sperm cells were analyzed by confocal microscopy using an Olympus FluoView TM FV1000 confocal microscope (Olympus, Argentina), with the FV10-ASW (version 01.07.00.16) software. Control (spermatozoa without TeNT-CPP), from capacitated and non-capacitated spermatozoa were included in all experiments. VAMP2 data were normalized subtracting the number of not stained cells in untreated samples (range 15–35% for capacitated cells and 20–40% for non-capacitated cells) and expressing the results of TeNT treated cells as a percentage of the untreated conditions. Differences between experimental and control conditions were tested by Parametric multiple comparison and Tukey–Kramer tests. Only significant differences ($p<0.05$) are discussed in the text.

SDS-PAGE and Western blot

The protein tyrosine phosphorylation profile of human spermatozoa (50×10^6) treated as explained in the legend of Fig. 3 were extracted in sample buffer and processed as described (Tomes *et al.*, 1998). Briefly, after removing the HTF by washing twice with PBS, sperm pellets were added to 6 × sample buffer (1 × final concentration: 2% SDS, 10% glycerol, 62.5 mM Tris–HCl) without disulfide reducing agents. Proteins were extracted by heating twice to 95 °C for 6 min each. Extracts were centrifuged (12,000 ***g***) for 10 min, and the supernatants were adjusted to 5% β-mercaptoethanol, boiled for 3 min, and used immediately or stored at −20 °C. Proteins were separated on 10% Tris-tricine-SDS gels and transferred to nitrocellulose membrane (Hybond-ECL; GE Healthcare, Buenos Aires, Argentina). Nonspecific reactivity was blocked by incubation for 1 h at RT in blocking solution (TBS/5% BSA, 0.1% Tween 20). Blots were incubated with primary mouse anti-PY antibody (0.2 μg/mL) in blocking solution for 1 h at RT. HRP-coupled goat anti-mouse was used as the secondary antibody (0.25 μg/mL) in blocking solution for 1 h at RT. Excess first and second antibodies were removed by washing five times for 10 min each in washing buffer (0.1% Tween 20 in TBS). Detection was accomplished with an enhanced chemiluminescence system (ECL; Amersham Biosciences, Buckinghamshire, UK) and visualized with a FujiFilm LAS-4000 Scanner (FujiFilm).

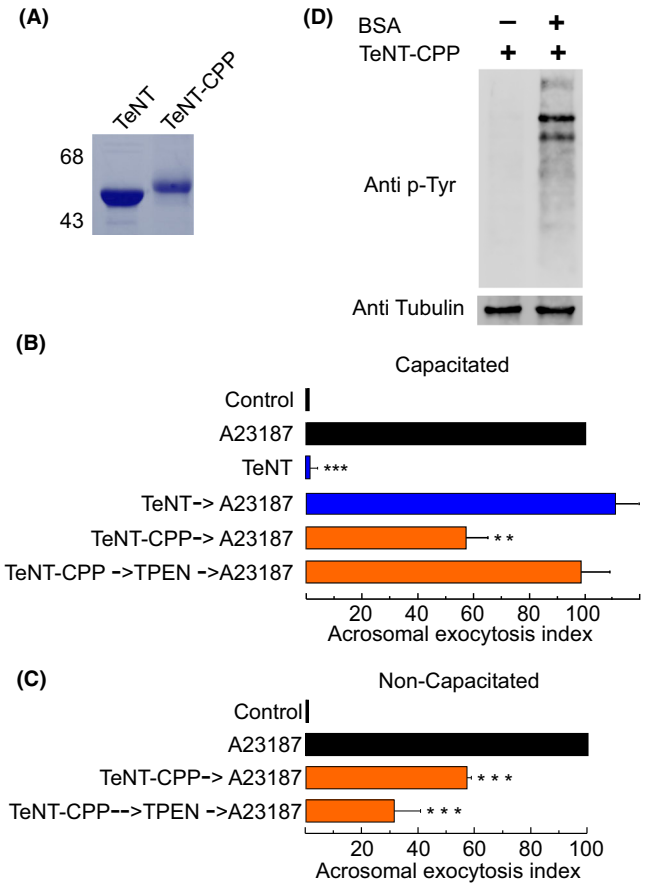
RESULTS

SNAREs of non-capacitated spermatozoa are sensitive to TeNT-CPP

So far, there is no evidence regarding the SNAREs configuration in non-capacitated human spermatozoa and the influences of capacitation on this molecular machinery. Our previous data showed that when capacitated spermatozoa were permeabilized and incubated in the presence of TeNT, calcium-induced acrosomal exocytosis was blocked. However, if the Zn^{2+} chelator, TPEN, was added after toxin incubation, but before the stimulation, calcium-induced acrosomal exocytosis was not inhibited. These observations suggest that in resting spermatozoa, SNAREs were assembled in toxin-resistant complexes that were disassembled after calcium activation. Testing the state of SNARE complexes in not permeabilized spermatozoa requires the engineering of a cell-permeant TeNT. For this purpose, the recombinant TeNT light chain was chemically cross-linked with a cell-penetrating peptide (RRRQRRKRRRQ). The peptide–protein conjugate was verified by SDS-PAGE. As shown in Fig. 1A, the rate electrophoretic mobility of TeNT-CPP was smaller compared with the not conjugated toxin, which is consistent with a larger molecular weight due to an efficient peptide/protein linkage. The membrane permeation and the cleavage activity of the TeNT-CPP were verified by incubation of capacitated spermatozoa with the toxin (30 min) followed by stimulation with the calcium ionophore A23187. The results showed a decrease in the acrosomal exocytosis index, indicating the TeNT was incorporated into the spermatozoa and was able to cleave VAMP2 upon stimulation. In contrast, spermatozoa incubated with TeNT-CPP (30 min), subsequently with TPEN (15 min, to inactivate the toxin) and then with A23187, reacted normally to the stimulation. These results indicate that the SNAREs were insensitive to TeNT before stimulation, and they sensitize upon stimulation of acrosomal exocytosis (Fig. 1B). These results are in full agreement with our previous data using permeabilized spermatozoa, confirming the existence of a cis-SNAREs configuration in resting capacitated spermatozoa that switches to a trans-SNARE configuration upon stimulation of acrosomal exocytosis, causing membranes bridging that promotes fusion (De Blas *et al.*, 2005). We then addressed the question about the SNARE conditions in non-capacitated spermatozoa. It has been previously reported that A23187 is capable of promoting mouse sperm capacitation (Tateno *et al.*, 2013). Therefore, we tested the action of A23187 on the acrosomal exocytosis of sperm recovered in HTF medium and incubated 3 h without BSA. Fig. 1C shows that the calcium ionophore was able to trigger acrosomal exocytosis in non-capacitated human spermatozoa. We then assessed the SNAREs configuration in non-capacitated spermatozoa. For this purpose, non-capacitated spermatozoa (recovered in HTF medium and incubated 3 h without BSA) were exposed to TeNT-CPP for

30 min followed by a subsequent incubation with or without TPEN to inactivate the toxin. The results show a decrease in the acrosomal exocytosis index even when the toxin was inactivated before the addition the calcium ionophore, supporting the idea that in the absence of albumin, the SNARE proteins were not assembled in neurotoxin-resistant complexes (Fig. 1C).

Figure 1 The acrosomal exocytosis was inhibited by TeNT-CPP in non-capacitated spermatozoa. (A) The recombinant TeNT was conjugated with a cell-penetrating peptide by crosslinking as described in Materials and Methods. The crosslinked process was checked by SDS-PAGE. TeNT conjugated with a polyarginine peptide (TeNT-CPP) shows less migration compared to not conjugated TeNT. Molecular weight standards (kDa) are indicated on the left. (B) Capacitated spermatozoa (5–10 $\times$ 10^6 cells/mL), and (C) non-capacitated spermatozoa were incubated during 30 min at 37 °C with 1 μM TeNT-CPP in HTF media, and the acrosomal reaction was induced with 10 μM of A23187 for 15 min. Alternatively, before triggering the acrosomal reaction, TeNT-CPP was inhibited with 25 μM of TPEN. A control of TeNT non-linked with CPP was used, showing none effect over the cell physiology (blue bars). The cells were fixed and stained with PSL-FITC. The percentage of reacted spermatozoa was normalized as described in Materials and Methods. Data represent the mean ± SEM of at least three independent experiments. The asterisks indicate significant differences (***$p < 0.001$; **$p < 0.01$) from positive controls. (D) After swim-up recovery in HTF medium, spermatozoa (30 $\times$ 10^6) were incubated 3 h at 37 °C with or without BSA plus 1 μM of TeNT-CPP. The cells were lysed and the protein tyrosine phosphorylation was detected with an anti-PY antibody. The left line shows the absence of any bands when cells were incubated with TeNT-CPP in the absence of BSA. The right line corresponding to cells incubated with TeNT-CPP and BSA shows the pattern of tyrosine-phosphorylated proteins, characteristic of capacitated spermatozoa. This image is representative of two independent experiments.

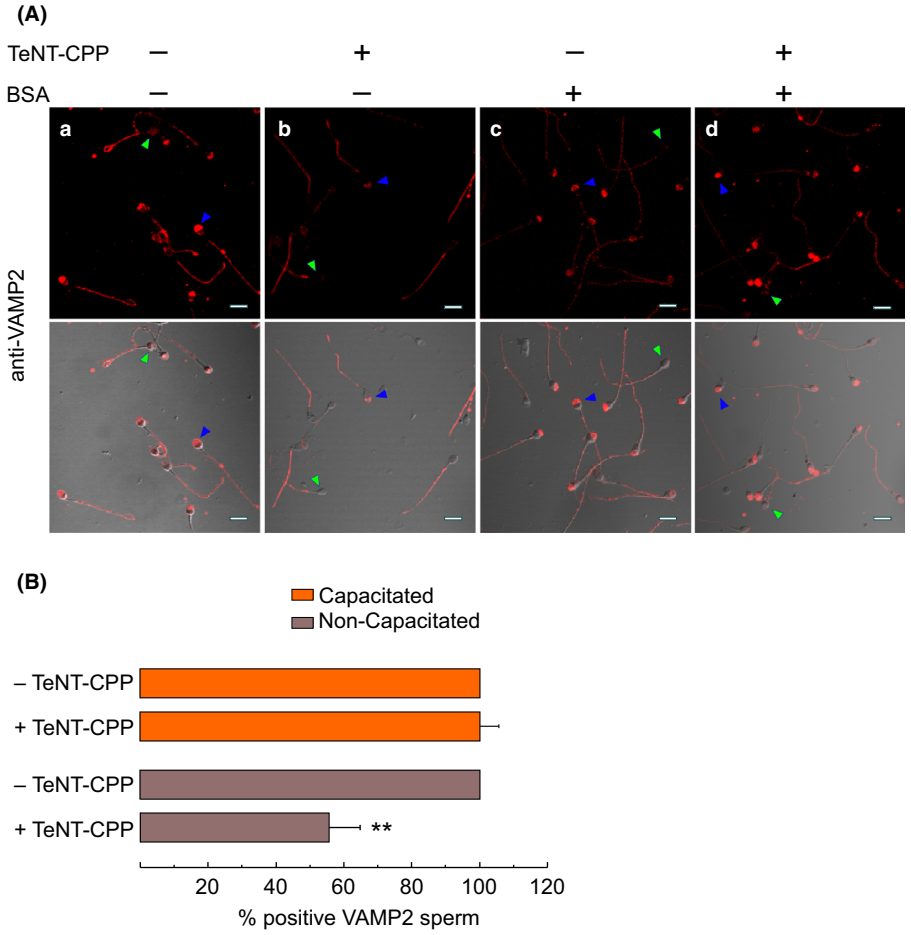


The functional assay relied on the essential role of VAMP2 for exocytosis. TeNT cleaves this protein when it is not associated with other SNARE proteins. An independent assay to measure VAMP2 integrity is by indirect immunofluorescence. We have previously reported that the immunoreactivity of the protein is lost when cleaved by the toxin (De Blas *et al.*, 2005). Therefore, detection of immunofluorescent signal indicates intact VAMP2, while the absence of this signal is indicative of cleaved VAMP2. Briefly, non-capacitated and capacitated spermatozoa were incubated with TeNT-CPP for 30 min. VAMP2 was detected by immunostaining with anti-VAMP2 antibody, followed by Cy3-conjugated fluorescent secondary antibody. The assay was evaluated by confocal microscopy, whereby unstained and stained cells were quantified. Incubation with TeNT-CPP decreased the percentage of stained cells in non-capacitated spermatozoa (Fig. 2A), whereas TeNT-CPP did not affect this parameter in capacitated spermatozoa (Fig. 2B). This indicates that SNAREs are in a toxin-sensitive configuration in non-capacitated spermatozoa and adopt a resistant configuration in BSA-capacitated spermatozoa. We then wondered whether capacitation could be affected by TeNT-CPP treatment. It has been well documented that the increase in global protein tyrosine phosphorylation is a hallmark of the capacitation process. The absence of any of the capacitation-factors (HCO_3^-, Ca^{2+}, or BSA) prevents both protein tyrosine phosphorylation and capacitation. For this purpose, we checked whether capacitation-dependent protein tyrosine phosphorylation is inhibited by TeNT-CPP added during capacitation. Non-capacitated spermatozoa were incubated with or without BSA in the presence of TeNT-CPP for 3 h. The cells were lysed and the tyrosine-phosphorylated proteins were detected by Western blot. Figure 1D shows normal tyrosine phosphorylation in BSA-capacitated spermatozoa even in the presence of the toxin. As expected, very low tyrosine phosphorylation was observed in spermatozoa incubated in the absence of BSA. Taken together all these data, we conclude that the VAMP2 was not affected by the presence of TeNT-CPP in capacitated spermatozoa, whereas, it was cleavage in non-capacitated spermatozoa, reflecting the disassembled SNAREs configuration before capacitation, vs. assembled SNAREs configuration after capacitation.

Non-capacitated spermatozoa incubated with TeNT-CPP and subsequently capacitated are unable to respond to progesterone

Because A23187 forces a Ca^{2+} increase and can cause capacitation and acrosomal exocytosis, we set out to evaluate the SNARE configuration in non-capacitated spermatozoa by triggering acrosomal exocytosis with progesterone, a physiological stimulant. It is well-known the progesterone response is one of the hallmarks of capacitation. Accordingly, acrosomal exocytosis should not be triggered in non-capacitated spermatozoa by progesterone. After the swim-up process, non-capacitated spermatozoa were co-incubated with TeNT-CPP and BSA. After 30 min, the TeNT-CPP activity was inhibited by TPEN, followed for 3 h and 15 min of incubation in the presence of BSA (represented as ---). At the end of the incubation, acrosomal exocytosis was triggered by progesterone stimulation. As shown in Fig. 3, a decrease in the acrosomal exocytosis index was observed when TeNT-CPP was added at the beginning of capacitation. In contrast, treatment with TeNT-CPP followed by TPEN did not affect

Figure 2 TeNT-CPP cleaved VAMP2 in non-capacitated spermatozoa. Capacitated and non-capacitated spermatozoa were incubated 30 min at 37 °C without or with 1 μM of TeNT-CPP. The cells were fixed and stained with the anti-VAMP2 antibody, followed by a secondary anti-mouse Cy3 antibody. (A) IFI showing images from the different experimental conditions: Non-capacitated without TeNT-CPP (a), non-capacitated with TeNT-CPP (b), capacitated without TeNT-CPP (c), and capacitated with TeNT-CPP (d). Spermatozoa were classified as VAMP2-stained (blue arrowhead) and VAMP2-not stained cells (green arrowhead). Bars, 5 μM. (B) At least 100 cells per condition were counted. The data were normalized as described in Materials and Methods. Data represent the mean ± SEM of three independent experiments. The asterisks indicate a significant difference (**$p < 0.01$) from controls spermatozoa without TeNT-CPP.



capacitated spermatozoa upon progesterone stimulation (Fig. 3). Thus, these results confirm that before or during the early stage of capacitation, the SNAREs are in a toxin-sensitive monomeric configuration and that, upon capacitation, they are subsequently assembled into a toxin-resistant configuration.

Kinetics of SNAREs assembly during capacitation

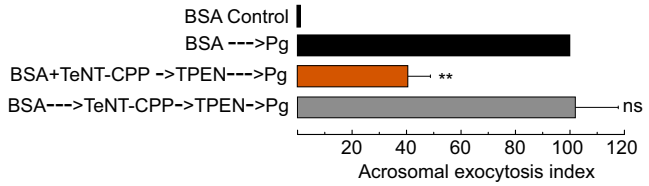
In the previous experiments, we showed that the SNARE proteins from non-capacitated spermatozoa are TeNT-CPP sensitive, and they exhibit a capacitation-dependent switch to a TeNT-CPP-resistant configuration. Moreover, we also showed that the tyrosine phosphorylation response was not modified by TeNT-CPP incubation, suggesting that capacitation was not blocked by the toxin. These observations and the possibility of using two membrane-permeant reagents, TeNT-CPP and TPEN, allow us to assess the time course of SNARE toxin sensitivity. For this assay, spermatozoa were recovered from swimming up in the absence of BSA and albumin was added at time zero, after which capacitation was followed for 4 h. At different timepoints of incubation, TeNT-CPP was added and, after 30 min incubation, the toxin was inactivated by TPEN (see the experimental setup in Fig. 4A). The results (Fig. 4B) showed that SNAREs became increasingly resistant to neurotoxin treatment with the capacitation time. Full protection was achieved after 2 h of incubation in the presence of BSA. Moreover, the experiment also shows that the cleavage of VAMP2 at early times of capacitation could not be rescued by later incubation in capacitation conditions, suggesting that spermatozoa only have a single set of SNARE proteins that are synchronically protected during capacitation.

DISCUSSION

Different events have been described during capacitation; however, many questions inherent to this process have not yet been addressed. One of the most studied events is the increase in tyrosine phosphorylated proteins, which depends on the presence of HCO_3^-, Ca^{2+}, and BSA; the absence of any of these factors prevents both tyrosine phosphorylation and capacitation.

The exocytosis of the acrosomal content, a mandatary event required for fertilization, is triggered by the fusion between the external acrosomal membrane and the plasma membrane, a process that requires a complex fusogenic machinery. It is well-

Figure 3 Progesterone fails to trigger acrosomal exocytosis when TeNT-CPP is added during the first 30 min of capacitation. After swim-up, spermatozoa (5–10 × 10^6 cells/mL) were resuspended with BSA and treated immediately (non-capacitated spermatozoa) or after 3 h 15 min (capacitated spermatozoa) with 1 μM of TeNT-CPP by 30 min, and subsequently with 25 μM of TPEN (gray bar). The acrosomal exocytosis was induced with 7 μM of progesterone for 30 min. Black bars indicate the negative (no progesterone stimulation) and positive (7 μM of progesterone during 30) controls. The cells were then fixed and stained with PSL-FITC. The percentage of reacted spermatozoa was normalized as described in Materials and Methods. Data represent the mean ± SEM of at least three independent experiments. The asterisks indicate significant differences (**$p < 0.01$) from positive controls.

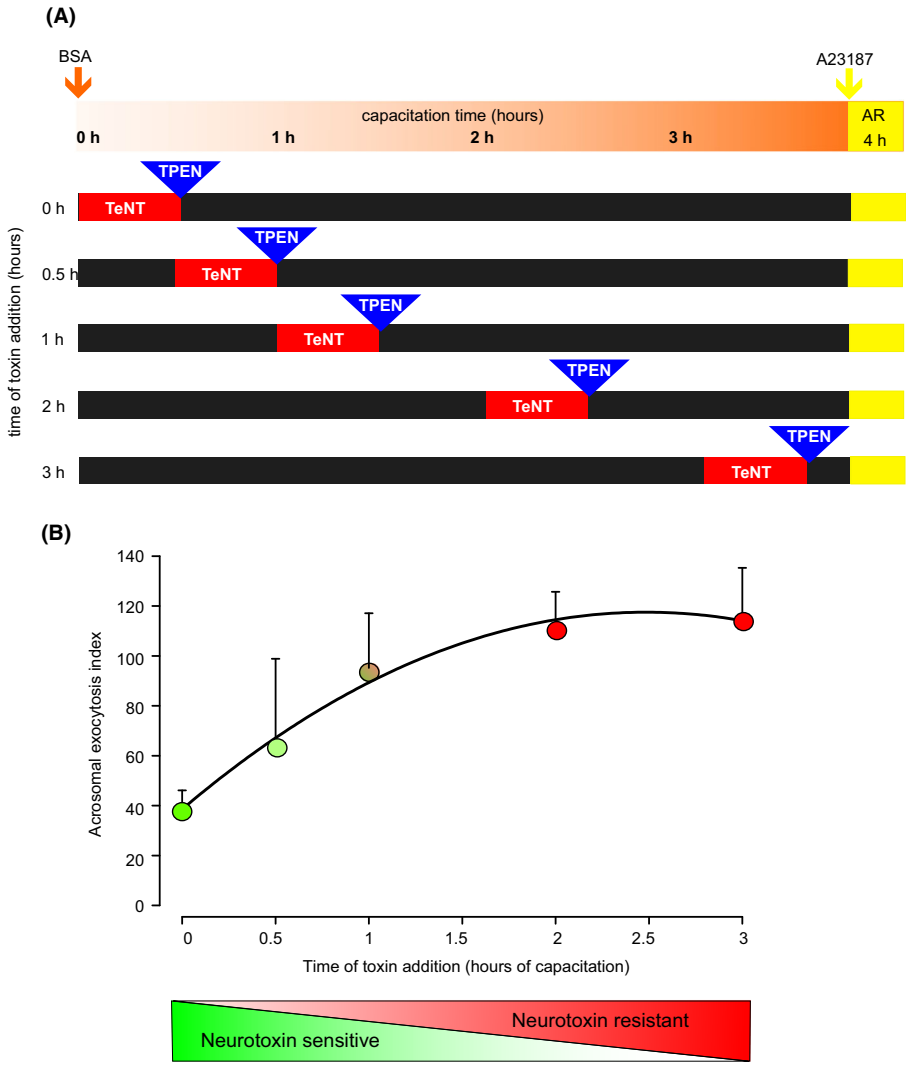


known that physiological stimuli can trigger acrosomal exocytosis only in capacitated spermatozoa. In this line, we wondered how the capacitation process influenced the SNAREs configuration and how this contributed to successful acrosomal exocytosis in human spermatozoa.

Tetanus and botulinum neurotoxins have been widely used to study the configuration of SNARE proteins. The light chain of tetanus toxin exhibits catalytic activity on VAMP2, cleaving this protein when it exists in the monomeric state and prevents the following: (A) SNAREs assembly, (B) membrane fusion, and (C) exocytosis.

We developed a powerful tool by linking the light chain of TeNT to a cell-permeable peptide, which permits access to the SNARE proteins in intact spermatozoa. The results reveal for the first time how capacitation is able to modulate the configuration of SNARE proteins in human spermatozoa. Capacitated spermatozoa showed normal levels of acrosome exocytosis when transiently exposed to TeNT-CPP, indicating the SNARE complexes

Figure 4 Kinetics of SNARE neurotoxin-resistance acquisition during capacitation. (A) Experimental design showing the addition time of TeNT-CPP (1 μM), TPEN (25 μM), and A23187 (10 μM) during capacitation in each condition. Capacitation was started by BSA addition (time 0 h). TeNT-CPP was added at different times of capacitation (0, 0.5, 1, 2, and 3 h), incubated for 30 min and then inhibited by TPEN. After 4 h of incubation, acrosome exocytosis was triggered by A23187 (15 min). Negative and positive controls were included in the assay. (B) Time-course showing the increase of acrosome exocytosis values as TeNT-CPP is added at later time points of capacitation. Data represent the mean ± SEM of four independent experiments.



are in a resistant configuration after capacitation, confirming our previous observations using SLO-permeabilized spermatozoa (De Blas *et al.*, 2005). Contrary, non-capacitated spermatozoa incubated with TeNT-CPP showed a decreased level of acrosomal exocytosis, which indicates that VAMP2 is in a monomeric toxin-sensitive configuration. An alternative read-out to study neurotoxin resistance of SNARE complexes is by detecting the VAMP2 integrity by IFI using an anti-VAMP2 antibody after TeNT-CPP treatment. Accordingly, the non-capacitated spermatozoa exposed to the neurotoxin had less VAMP2 labeling (indicating VAMP2 cleavage), while the capacitated spermatozoa had increased antibody staining (indicating intact, functional VAMP2). Similarly, a physiological stimulant like progesterone failed to induce acrosomal exocytosis when spermatozoa were transiently treated with TeNT-CPP at early times during capacitation. In contrast, the treatment did not prevent exocytosis in fully capacitated spermatozoa.

The kinetic assays showed that full protection against the neurotoxin was achieved after 2 h of capacitation. According to the results shown in Figs 3 and 4, the early cleavage of VAMP2 could not be recovered by post-incubation with BSA. This observation supports the idea that spermatozoa have a limited number of SNAREs in storage, and they need to be ready for membrane fusion when exocytosis is stimulated. This is congruent with the fact that sperm cells have a single opportunity to undergo the acrosome reaction.

Normal tyrosine phosphorylation was observed in spermatozoa capacitated in the presence of TeNT-CPP, suggesting TeNT-CPP does not prevent other capacitation-associated events. In this regard, we have recently published the action of Corza6 (C6), a peptide with inhibitory activity over hHv1 (human voltage-gated proton channel) (Zhao *et al.*, 2018). This channel extrudes H^+ during capacitation in human spermatozoa. Capacitation in the presence of C6 inhibits acrosomal exocytosis, but does not block tyrosine phosphorylation (Zhao *et al.*, 2018).

We speculate two possible sceneries in non-capacitated spermatozoa: *i)* SNAREs switching constantly between disassembled (monomeric, TeNT sensitive) and assembled (TeNT resistant) configurations or *ii)* static monomeric SNARE configuration, available to TeNT cleavage. We favor the first possibility since our laboratory has previously shown that NSF—a chaperone protein required for disassembling *cis* SNARE complexes—is inactivated by tyrosine phosphorylation in capacitated spermatozoa (Zarelli *et al.*, 2009). It is tempting to speculate this phosphorylation occurs during capacitation. Hence, in non-capacitated spermatozoa, NSF could be active, maintaining VAMP2 in a dynamic equilibrium between protected and unprotected configurations, while in capacitated spermatozoa when NSF is inactive, VAMP2 is maintained in protected complexes. Finally, when the acrosome reaction is triggered, NSF dephosphorylated by PTP1b phosphatase. promoting the disassembly of the SNARE complexes and rendering them able to re-assembly in trans-competent complexes to fuse the membranes.

The capacitation process promotes a multiplicity of events, all necessary to fine-tune the sperm-egg interaction. Based on the findings herein, SNARE stabilization in cis complexes that is achieved during capacitation is a necessary event that prepares the immature spermatozoa for a successful encounter with the oocyte.

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DISCLOSURES

The authors have no conflict of interest to disclose.

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