HHS Public Access

Author manuscript

J Cell Physiol. Author manuscript; available in PMC 2019 December 01.

Published in final edited form as:

J Cell Physiol. 2018 December ; 233(12): 9685–9700. doi:10.1002/jcp.26883.

Only a subpopulation of mouse sperm displays a rapid increase in intracellular calcium during capacitation

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Abstract

Mammalian sperm must undergo a functionally defined process called capacitation to be able to fertilize oocytes. They become capacitated *in vivo* by interacting with the female reproductive tract or *in vitro* in a defined capacitation media that contains bovine serum albumin (BSA), calcium (Ca^{2+}) and bicarbonate (HCO_3^-). In this work, sperm were double stained with propidium iodide and the Ca^{2+} dye Fluo-4 AM and analyzed by flow cytometry to determine changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). An increase in $[Ca^{2+}]_i$ was observed in a subpopulation of capacitated live sperm when compared with non-capacitated ones. Sperm exposed to capacitating medium displayed a rapid increase in $[Ca^{2+}]_i$ within 1 min of incubation, which remained sustained for 90 min. These rise in $[Ca^{2+}]_i$ after 90 min of incubation in capacitating medium was evidenced by an increase in the normalized median fluorescence intensity (MFI). This increase was dependent on the presence of extracellular Ca^{2+} and at least in part reflected the contribution of a new subpopulation of sperm with higher $[Ca^{2+}]_i$. In addition, it was determined that the capacitation-associated $[Ca^{2+}]_i$ increase was dependent of CatSper channels, as sperm derived from CatSper knockout (CatSper KO) or incubated in the presence of CatSper inhibitors failed to increase $[Ca^{2+}]_i$. Surprisingly, a minimum increase in $[Ca^{2+}]_i$ was also observed in CatSper KO sperm suggesting the existence of other Ca^{2+} transport system. Altogether, these results indicate that a subpopulation of sperm increases $[Ca^{2+}]_i$ very rapidly during capacitation mainly due to a CatSper-mediated influx of extracellular Ca^{2+}.

Keywords

Sperm; Calcium; Capacitation; CatSper

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Introduction

Freshly ejaculated mammalian sperm do not have the ability to fertilize oocytes. They must undergo a functionally defined process called capacitation (Chang, 1951; Austin, 1952), which allows them to develop hyperactivated motility and the ability to undergo acrosomal exocytosis (Suarez, 2008; Buffone, Hirohashi and Gerton, 2014). Sperm become capacitated *in vivo* by interacting with the female reproductive tract or *in vitro* in a defined capacitation media that contains bovine serum albumin (BSA), calcium (Ca^{2+}) and bicarbonate (HCO_3^-) (Yanagimachi, 1994; Visconti, Bailey, *et al.*, 1995). From a molecular point of view, capacitation leads to an increase in cAMP, Protein Kinase A (PKA, aka cAMP-dependent protein kinase) activity and tyrosine phosphorylation of sperm proteins (Visconti, Moore, *et al.*, 1995; Alvau *et al.*, 2016), membrane hyperpolarization (De La Vega-Beltran *et al.*, 2012) as well as a rise in intracellular pH (pHi) (Stival *et al.*, 2016) and in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Ruknudin and Silver, 1990). The increase in $[Ca^{2+}]_i$ was shown to be essential for hyperactivation and acrosomal exocytosis in several mammalian species (Marín-Briggiler *et al.*, 2003; Darszon *et al.*, 2011; Correia, Michelangeli and Publicover, 2015). The predominant source of Ca^{2+} during hyperactivation in the female genital tract is extracellular Ca^{2+} that enters through CatSper channels (Kirichok, Navarro and Clapham, 2006). CatSper proteins are only expressed in male germ cells and localize specifically to the plasma membrane of the principal piece of the flagellum in mature sperm (Carlson *et al.*, 2005; Jin *et al.*, 2007). Male mice lacking any of the CatSper1–4 genes are infertile and their sperm are unable to undergo hyperactivation (Ren *et al.*, 2001; Quill *et al.*, 2003; Qi *et al.*, 2007; Chung *et al.*, 2011), as are human males with loss-of-function mutations (Avidan *et al.*, 2003; Avenarius *et al.*, 2009; Smith *et al.*, 2013).

Most of our knowledge about capacitation comes from *in vitro* experiments using whole sperm populations, which derive from average responses of sperm with different degrees of capacitation, that consider not only normal cells but also dead as well as abnormal gametes. Previous observations by multiple laboratories indicate that sperm population is not homogeneous and only a subset of sperm undergoes capacitation (Buffone *et al.*, 2004; Escoffier *et al.*, 2015). Sperm $[Ca^{2+}]_i$ is usually assessed using Ca^{2+} sensitive probes such as Fluo and Fura by fluorometry (Mata-Martínez *et al.*, 2013), flow cytometry (López-González *et al.*, 2014) and single-cell analysis (Romarowski *et al.*, 2016). Single cell analysis has the advantage of studying molecular events in individual sperm and detecting local changes in different sperm compartments. However, this method is not suitable for high-throughput studies. On the other hand, flow cytometry can analyze thousands of cells very rapidly compared to the time required for single-cell studies, and results in a valuable tool for the analysis of different populations of capacitated sperm (Tao *et al.*, 1993; Piehler *et al.*, 2006; Robles and Martínez-Pastor, 2013). Using this approach, it was reported how changes in sodium (Escoffier *et al.*, 2012), membrane potential (Escoffier *et al.*, 2015), membrane fluidity (Gadella *et al.*, 1999), pH (López-González *et al.*, 2014; Puga Molina *et al.*, 2017) and acrosomal exocytosis (Zoppino *et al.*, 2012; Hirohashi *et al.*, 2015; Romarowski *et al.*, 2015) occur in individual sperm during capacitation. However, despite the importance of Ca^{2+} fluxes in events leading to capacitation, analysis of $[Ca^{2+}]_i$ in mouse sperm is still scarce.

In this study, we analyzed $[Ca^{2+}]_i$ changes during capacitation from epididymal mouse sperm using flow cytometry. We observed that there is a subpopulation of sperm that increases $[Ca^{2+}]_i$ very rapidly during capacitation due to an influx from extracellular Ca^{2+}. The rise in $[Ca^{2+}]_i$ appears to be controlled by HCO_3^- and BSA present in the capacitation media and is mediated by CatSper channels. However, our experiments also suggest the presence of a CatSper-independent Ca^{2+} influx that could be critical for sperm capacitation.

Materials and Methods

Reagents

Chemicals were obtained from the following sources: bovine serum albumin (BSA) A7906, Ca^{2+} ionophore A23187, Mibefradil, NNC55-0396 and Ethylene glycol-bis (2-aminoethylether)-N,N,N,N′tetraacetic acid (EGTA) were purchased from Sigma–Aldrich Chemical Co. (St.Louis, MO); Fluo-4 AM and Fluo-3 AM from Molecular Probes, Thermo Fisher Scientific; Pluronic acid from Life Technologies Corporation (Invitrogen); PI from Santa Cruz (Santa Cruz, USA) and Ionomycin from Alomone Labs (Jerusalem, Israel). All other chemicals were of reagent grade. Fluo-4 AM, Fluo-3 AM, Pluronic acid, Ca^{2+} ionophore A23187 and Ionomycin were dissolved in DMSO; EGTA was dissolved in non-capacitating modified TYH medium without Ca^{2+} (-HCO_3^-, -BSA, -Ca^{2+}); while PI, Mibefradil and NNC55-0396 were dissolved in hexa-distilled water.

Animals

Hybrid F1 (C57BL/6 x Balb/C) mature (10–12 weeks-old) male mice were used. CatSper KO (Ren *et al.*, 2001) mice and their corresponding wild-type siblings were on a mixed background (C57BL/6 and CD1), and were provided by Dr. Pablo Visconti from University of Massachusetts (Navarrete *et al.*, 2016). CatSper-null mice were euthanized in accordance with the Animal Care and Use Committee (IACUC) guidelines of UMass-Amherst. In all cases, mice were housed in groups of 4 or 5 in a temperature-controlled room (23°C) with lights on at 07:00 h and off at 19:00 h, and had free access to tap water and laboratory chow. All experimental procedures were carried according to guidelines of the institutional animal care and were reviewed and approved by the Ethical Committee of the *Instituto de Biología y Medicina Experimental, Buenos Aires*. Experiments were performed in strict accordance with the Guide for Care and Use of Laboratory Animals approved by the National Institutes of Health (NIH).

Sperm medium

The non-capacitating medium used in this study was a modified Toyoda–Yokoyama–Hosi (modified TYH) containing 119.3 mM NaCl, 4.7 mM KCl, 1.71 mM $CaCl_2.2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 0.51 mM sodium pyruvate, 5.56 mM glucose, 20 mM HEPES, 10 μg/ml gentamicin and phenol red 0.0006% (NC medium). For capacitating conditions 15 mM $NaHCO_3$ and 5 mg/ml BSA were added (CAP medium). In the cases where medium without added Ca^{2+} salts was required, 1.71 mM $CaCl_2.2H_2O$ was omitted (nominal zero Ca^{2+} medium, indicated by Ca^{2+}=0). When chelation of the extracellular Ca^{2+} was needed, we added 1 mM EGTA at nominal zero Ca^{2+} medium. In all cases, pH was adjusted to 7.4 with NaOH.

Sperm capacitation

Animals were euthanized and cauda epididymal mouse sperm were collected. Both cauda epididymis were placed in 1 ml of non-capacitating modified TYH medium (without BSA and $NaHCO_3$) in the presence or absence of Ca^{2+} as described for each experiment. After 15 min of incubation at 37 °C (swim-out), epididymis were removed, and sperm were resuspended to a final maximum concentration of $1x10^7$ cells/ml on 100 µl of the appropriate medium depending on the experiment performed. A 10 min pre-incubation in 100 µl of non-capacitating media containing CatSper inhibitors (30 µM Mibefradil or 10 µM NNC55-0396) was done when required. An equal volume (100 µl) of non-capacitating medium or two-fold concentrated capacitating media (30 mM $NaHCO_3$ and 10 mg/ml BSA) was added, and sperm were incubated for different time periods at 37°C.

Determination of $[Ca^{2+}]_i$ by flow cytometry

Sperm $[Ca^{2+}]_i$ changes were assessed using Fluo-4 AM. After incubation in the appropriate medium, samples were centrifuged at 400 x *g* for 4 min at room temperature and resuspended in 200 µl of non-capacitating media containing 1 µM Fluo-4 AM and 0.02% Pluronic acid for 20 min at 37°C. Samples were washed again and resuspended in 50 µl of non-capacitating TYH media. Before collecting data 2 ng/µl of PI was added to monitor viability. In some experiments 10 µM of Ca^{2+} ionophore A23187 was also added to the samples. Data were recorded as individual cellular events using a BD LSRFortessa TM cytometer (Biosciences; Becton, Dickinson and Company) for the experiments involving CatSper KO and their wild-type mice siblings and BD FACSCanto II TM cytometer (Biosciences; Becton, Dickinson and Company) for all other experiments. Side-scatter area (SSC-A) and forward-scatter area (FSC-A) data were collected from 20,000 events per sample in order to define sperm population as previously described (Escoffier *et al.*, 2012). In all cases, doublet exclusion was performed analyzing two-dimensional dot plot FSC-A vs. forward-scatter height (FSC-H). Doublets exhibit a higher signal width or area to height ratio compared to single cells (singlets). Events deviating from the diagonal are doublets. Positive cells for Fluo-4 AM were collected using the filter for Fluorescein isothiocyanate (FITC; 530/30), and for PI the filter for Peridinin chlorophyll protein complex PerCP (670LP). The two indicators had minimal emission overlap, but still compensation was done. Data were analyzed using FlowJo software (V10.0.7).

Live imaging of $[Ca^{2+}]_i$ in individual mouse sperm

Sperm were incubated with 2 µM Fluo-3 AM and 0.05% Pluronic acid in non-capacitating TYH modified medium with BSA (5 mg/ml) during 20 min. Once loaded, the cells were centrifuged at 500 x *g* for 4 min at room temperature and resuspended in 200 µl of non-capacitating media with BSA. Sperm were immobilized on mouse laminin (0.1 mg/ml) coated-cover slips to allow recordings. The chamber was filled with recording medium (non-capacitating TYH). Ca^{2+} imaging was performed before, during, and after TYH + 30 mM HCO_3^- or non-capacitating TYH (vehicle) addition. At the end of each recording, 10 µM Ionomicyn was added to assess the viability of the cells. Those cells that did not display a robust Ca^{2+} response after addition of Ionomicyn were not included in our analysis. The images were taken in a Zeiss LSM880 scan head on an axio observer Z1 inverted

microscope with a 60x1.4 AN oil immersion objective. A laser line 488 nm of an argon ion laser was used for the excitation, while the detection was done in a GaAsP spectral detector with a bandwidth between 508 and 588 nm. Bi-directional scanning was used with a Dwell time of 1.03 μsec at 512x512 pixels resulting in an acquisition of 3 frames per second for periods of 8–10 minutes. Movies were processed and analyzed with Image J (v1.38, NIH). Regions of interest (ROIs) were drawn on each sperm for fluorescence quantification. Intracellular [Ca^{2+}] is presented as (F-F0)/F0 ratios after background subtraction, where F-F0 was the change in fluorescence signal intensity and F0 was the baseline as calculated by averaging 180 frames before stimulus application.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) of at least four independent experiments for all determinations. Statistical analyses were performed using the GraphPad Prism 6 software (La Jolla, CA USA). The differences between means of only 2 groups were analyzed by paired t-test (single-cell analysis). Two-way analysis of variance (ANOVA) with repeated measures was used to analyze the effect of incubation medium in normalized median fluorescence intensity (MFI) of Fluo-4 AM and percentage of sperm with high Fluo-4 AM fluorescence, over time. One-way ANOVA for matched data was performed to analyze percentage of sperm with high Fluo-4 AM fluorescence comparing different incubation conditions, while non-parametric Friedman test was performed in combination with Dunn's multiple comparisons test to analyze normalized MFI of Fluo-4 AM. Two-way ANOVA for independent measures was performed to analyze normalized MFI of Fluo-4 AM and percentage of sperm with high Fluo-4 AM fluorescence, for the effects of: experimental condition x genotype. Post hoc Sidak's test was employed when necessary. A probability (p) value $p<0.05$ was considered statistically significant. Parametric or non-parametric comparisons were used as dictated by data distribution.

Results

Only a subpopulation of capacitated mouse sperm displays an increase in $[Ca^{2+}]_i$

Intracellular [Ca^{2+}] was measured in individual cells using flow cytometry. Sperm were loaded with the Ca^{2+} probe Fluo-4 AM and PI to differentiate between live and dead sperm. Briefly, the sperm population was defined as previously described (Escoffier *et al.*, 2012), where 0.1% Triton X-100 was used to discriminate non-sperm particles passing through the flow cytometer detector as it solubilizes non-sperm particles. Then those sperm associated in doublets were excluded. Once non-sperm events and sperm doublets were gated out (Figure 1A–B), two-dimensional fluorescence dot plots of Fluo-4 AM (positively correlated with $[Ca^{2+}]_i$) versus PI (to label DNA of dying cells) were created. In order to make compensation, three controls were performed for each experiment: without any staining (-Fluo-4 AM / -PI), with only PI staining (-Fluo-4 AM / +PI), or with only Fluo-4 AM staining (+Fluo-4 AM / -PI) (Figure 1C). In experimental samples, by using the two-dimensional fluorescence dot plots, the live sperm population was gated (negative for PI staining). This population was used for the analysis of $[Ca^{2+}]_i$ by performing histograms that display fluorescence of Fluo-4 AM on the x-axis and normalized frequency of sperm on the y-axis (Figure 1D). Both, two-dimensional dot plots and histograms, were used to observe

changes in $[Ca^{2+}]_i$ of live sperm. An increase in Fluo-4 AM fluorescence was observed in live sperm incubated with Ca^{2+} ionophore A23187 (Figure 1D right) when compared with non-treated ones (Figure 1D left). This ionophore-induced increase did not occur in dead sperm (PI positive, Figure 1D).

To investigate the effect of capacitation in the regulation of $[Ca^{2+}]_i$, Fluo-4 AM fluorescence was analyzed in sperm treated with three different conditions. First, $[Ca^{2+}]_i$ was determined immediately after recovering sperm from the swim-out (grey shadow in Figure 2A); second, the analysis was conducted in sperm incubated for 90 min in conditions that do not support capacitation; and finally, $[Ca^{2+}]_i$ was assessed in sperm incubated for 90 min in capacitating medium (Figure 2A). Incubation for 90 min in capacitating medium led to an increase in normalized MFI of Fluo-4 AM in live sperm, which was not observed in non-capacitating conditions (Figure 2B). Interestingly, incubation for 90 min in both, non-capacitating and capacitating conditions led to an increase in Fluo-4 AM fluorescence in a live sperm subpopulation, which can be observed in the dot plots, when compared with those recovered from the swim-out (Figure 2C, upper panels). Once again, this $[Ca^{2+}]_i$ increase did not occur in dead sperm (PI positive, Figure 2C, upper panels). However, the percentage of sperm with high $[Ca^{2+}]_i$ was significantly higher in the capacitated population. The increase can be also visualized using normalized frequency histograms (Figure 2C, bottom panels). The percentage of sperm undergoing $[Ca^{2+}]_i$ increase was plotted for sperm from 12 different mice (Figure 2D). In every case the population of sperm with high fluorescence was determined from the capacitating control condition (CAP 90 min) and extrapolated to the others conditions of each experiment (Figure 2C).

The capacitation-induced increase in $[Ca^{2+}]_i$ can be due to an influx from extracellular Ca^{2+} and/or to the release of this ion from sperm intracellular Ca^{2+} storages (e.g. acrosome, redundant nuclear envelope). To elucidate the source of Ca^{2+}, Fluo-4 AM fluorescence was analyzed in sperm incubated in Ca^{2+}-free medium (nominal zero Ca^{2+} concentration, Ca^{2+}=0) or in Ca^{2+}-free medium with the addition of 1 mM EGTA (Ca^{2+}=0 + EGTA), given that Ca^{2+}=0 media still contain contaminant Ca^{2+} at micromolar concentrations (Marin-Briggiler, 2005). In neither of these conditions the increase in $[Ca^{2+}]_i$ in non-capacitating and capacitating media was observed (Figure 3A). These results support the hypothesis that the presence of extracellular Ca^{2+} is required for the capacitation-associated increase in $[Ca^{2+}]_i$. On the other hand, as a positive control, and to determine the higher sensitivity limit of our assay, sperm were exposed to A23187. In the presence of Ca^{2+} in the incubation media, addition of Ca^{2+} ionophore resulted in an increase in $[Ca^{2+}]_i$ in every case (Figure 3B) which was not observed when A23187 was added to sperm incubated in Ca^{2+}=0 media (Figure 3C). Previous reports indicate that Ca^{2+} ionophore A23187 may be used to release Ca^{2+} from intracellular stores in the absence of extracellular Ca^{2+} in other cell types (Pelassy, Breittmayer and Aussel, 1992; Rzigalinski, Blackmore and Rosenthal, 1996; Cobbold, Brookes and Wileman, 2000). However, the absence of $[Ca^{2+}]_i$ increase with A23187 in Ca^{2+}=0 media indicated that no mobilization of Ca^{2+} from intracellular stores was induced in sperm (Figure 3C).

The increase in $[Ca^{2+}]_i$ is an early capacitation event and is independently regulated by HCO_3^- and BSA

Sperm capacitation is a time-dependent process; while some signaling pathways are activated as soon as the sperm encounters a capacitation-supporting medium, others only occur after several minutes to hours of incubation. To test the kinetics of Ca^{2+} changes, $[Ca^{2+}]_i$ was analyzed in sperm incubated in medium that supports capacitation or not, at different time points. In capacitating conditions, a very rapid rise in $[Ca^{2+}]_i$ starting at 1 min of incubation was observed in a sperm subpopulation (Figure 4A). The percentage of sperm with high Ca^{2+} remains stable for at least 90 min. On the other hand, in non-capacitating conditions, although a percentage of the sperm population also presented higher $[Ca^{2+}]_i$, the relative amount of $[Ca^{2+}]_i$ was much lower. The changes in the normalized MFI of Fluo-4 AM and the percentage of sperm that belongs to the responsive population at different time points during capacitation are plotted for 4 independent experiments (Figure 4B and 4C, respectively).

In our experimental conditions, the difference between the medium that supports capacitation and the one that does not is the presence of BSA and HCO_3^-. To test the participation of these molecules in the capacitation-associated $[Ca^{2+}]_i$ increase independently, sperm were incubated for 90 min in the presence or absence of one or both compounds. Incubation with HCO_3^- alone stimulated an increase in the percentage of sperm with high Ca^{2+} in comparison with sperm incubated in medium that did not support capacitation (-HCO_3^-, -BSA, +Ca^{2+}). On the other hand, BSA had an even greater effect stimulating a rise in $[Ca^{2+}]_i$ to similar levels as those obtained in the presence of both, HCO_3^- and BSA (Figure 5A–C). The effect of BSA in the regulation of $[Ca^{2+}]_i$ has been analyzed previously in single cells (Xia and Ren, 2009). The flow cytometry experiments suggest that HCO_3^- alone is also able to induce elevation of $[Ca^{2+}]_i$. To further test this hypothesis, changes in $[Ca^{2+}]_i$ were analyzed using single cell imaging of Fluo-3 AM-loaded sperm. Addition of HCO_3^- resulted in a rapid $[Ca^{2+}]_i$ increase (Figure 5E and F) which did not occur when non-capacitating TYH medium (vehicle) was added (Figure 5D and F, and Supplementary Movies S1–S2). Overall, flow cytometry and single cell analyses suggest that both BSA and HCO_3^- contribute to the increase in $[Ca^{2+}]_i$ observed under capacitating conditions.

CatSper activity is required for the capacitation-associated $[Ca^{2+}]_i$ increase

To assess the participation of CatSper, $[Ca^{2+}]_i$ was evaluated in the presence or absence of voltage-gated Ca^{2+} channel (Cav) blockers: Mibefradil and NNC55-0396 (Lishko, Botchkina and Kirichok, 2011; Strünker *et al.*, 2011). Sperm were incubated for 90 min in capacitating conditions in the presence of 30 μM Mibefradil or 10 μM NNC55-0396, concentrations previously shown to block CatSper currents using electrophysiological measurements (Strünker *et al.*, 2011). These concentrations did not affect sperm viability (data not shown). Addition of these compounds suppressed the capacitation-associated $[Ca^{2+}]_i$ increase (Figure 6). The inhibition can be visualized by a decrease in the normalized MFI of Fluo-4 AM (Figure 6B) as well as in the percentage of sperm that displayed a rise in $[Ca^{2+}]_i$ (Figure 6C).

To further test the role of CatSper channels we used CatSper-null sperm. Intracellular [Ca^{2+}] analysis was performed in CatSper KO sperm and compared with sperm from wild-type siblings. The absence of functional CatSper channel displayed a significant decrease in $[Ca^{2+}]_i$ in comparison with the rise observed in wild-type mice (Figure 7A), which can also be observed by a decrease in the normalized MFI of Fluo-4 AM (Figure 7B) and in the percentage of sperm that displayed a rise in $[Ca^{2+}]_i$ (Figure 7C). Interestingly, in CatSper KO mice, incubation for 90 min in capacitating media led to a slight increase of $[Ca^{2+}]_i$ as determined by normalized MFI of Fluo-4 AM ($p=0.073$; Figure 7B) and by the percentage of sperm with high Fluo-4 AM fluorescence ($p=0.051$; Figure 7C) in comparison with non-capacitating cells (Figure 7). This result suggests that, even in the absence of CatSper, there is a minimum increase in $[Ca^{2+}]_i$ during capacitation.

Discussion

After ejaculation, sperm are able to move actively but lack fertilizing competence. They acquire the ability to fertilize in the female genital tract in a time-dependent process called capacitation (Austin, 1951; Chang, 1951). Initially, capacitation was defined using fertilization as an end-point. However, a variety of evidence suggests that the functional changes occurring in the sperm during capacitation are not one event, but a combination of sequential and concomitant signaling processes (Visconti *et al.*, 2011) that include complex signaling cascades where intracellular Ca^{2+} plays a central role. The participation of Ca^{2+} in the regulation of capacitation process is well documented (Darszon *et al.*, 2011; Visconti *et al.*, 2011). It was clearly observed when sperm were incubated in Ca^{2+}-free media, either with or without chelating agents such as EGTA (Ahmad *et al.*, 1995; Marquez, Ignotz and Suarez, 2007; Torres-Flores *et al.*, 2011; Battistone *et al.*, 2014) or by elevating its levels using Ca^{2+} ionophores like A23187 (Tateno *et al.*, 2013). In addition, pharmacological and genetic loss-of-function experiments have shown a central role of Ca^{2+} in the regulation of sperm motility, hyperactivation and acrosomal exocytosis (Suarez and Dai, 1995; Ho and Suarez, 2001; Darszon *et al.*, 2011).

One of the first events that triggers sperm capacitation is the activation of a cAMP pathway (Buffone *et al.*, 2014) after sperm are released from the epididymis and interact with higher HCO_3^- and Ca^{2+} concentrations present in the seminal fluid and the female reproductive tract (Okamura *et al.*, 1985). Genetic loss-of-function experiments demonstrated the essential role of proteins involved in the cAMP pathway (Hess *et al.*, 2005) in sperm capacitation and fertilization. In agreement with the role of Ca^{2+} in these events, mouse sperm exposed to the Ca^{2+} ionophore A23187 are able to develop hyperactivation, undergo acrosomal exocytosis and acquire fertilizing ability even when the cAMP pathway is completely inhibited (Tateno *et al.*, 2013). In addition, it is considered that Ca^{2+} has a biphasic role in sperm capacitation signaling pathways (Navarrete *et al.*, 2015). Incubation of mouse sperm in the absence of added extracellular Ca^{2+} blocked the capacitation-associated increase in tyrosine phosphorylation (Marin-Briggiler, 2005). However, addition of EGTA to further lower the extracellular Ca^{2+} (traces at micromolar concentrations) promotes a strong increase in tyrosine phosphorylation. A similar effect was also observed when adding calmodulin antagonists or calcineurin inhibitor (Navarrete *et al.*, 2015). These results suggest that Ca^{2+} ions regulate sperm cAMP and tyrosine phosphorylation pathways

in a biphasic manner and that some of its effects are mediated by calmodulin (Navarrete *et al.*, 2015).

Despite all this evidence, there are few reports that directly measure Ca^{2+} levels during capacitation in mammalian sperm (Jai *et al.*, 1978; Coronel and Lardy, 1987; White and Aitken, 1989; Ruknudin and Silver, 1990; Zhou *et al.*, 1990; Baldi *et al.*, 1991). Ruknudin and Silver reported an increase in $[Ca^{2+}]_i$ during mouse sperm capacitation (Ruknudin and Silver, 1990). However, their approach relied on the use of Ca^{2+}-selective microelectrodes to measure changes in $[Ca^{2+}]_i$ (Ca^{2+} uptake). Although it was a reasonable method at that time, these experiments have two major disadvantages: first, the low sensitivity of the method itself; and second, the fact that it only registers changes in the $[Ca^{2+}]$ present in the incubation media. Another attempt to measure Ca^{2+} was using single cell approaches. However, the time frame of these experiments preclude to evaluate a large number of cells and require sophisticated equipment. We decided to use flow cytometry because it allows the analysis of thousands of cells within seconds resulting in a very useful method to evaluate single cell behavior in a much larger population. Furthermore, a distinction between live and dead sperm can be performed by the addition of viability dyes. Most studies on sperm capacitation have been done with complete sperm suspensions containing sperm in different stages of capacitation, as well as deteriorated sperm cells (Huszar *et al.*, 1998; Buffone *et al.*, 2004; García-Álvarez *et al.*, 2014). On the other hand, different studies have revealed that capacitation generates heterogeneous sperm populations, highlighting the importance of evaluating single cell behavior (Escoffier *et al.*, 2012, 2015; Romarowski *et al.*, 2016). The reason for this heterogeneity is unknown but may be originated from different epididymal maturation states. It is also hypothesized that having sperm at different stages of capacitation could extend the time period in which they are capable to fertilize an ovulated egg.

In this work, flow cytometry was used to analyze changes in $[Ca^{2+}]_i$ during capacitation. For this purpose, sperm suspensions were double stained with the Ca^{2+} dye Fluo-4 AM in combination with PI to analyze these changes in individual live sperm. We observed that only a subpopulation of the sperm incubated under capacitating conditions effectively responds displaying a rapid increase in $[Ca^{2+}]_i$ within 1 minute. Our results indicate that sperm incubated in media that do not support capacitation are constituted by mainly two subpopulations. In contrast, sperm incubated under capacitating conditions distributed in three defined subpopulations, where two display similar Fluo-4 AM average fluorescence than the non-capacitated sperm population, while the other one depicted a higher fluorescence.

Two Ca^{2+} transport systems were clearly identified in mammalian sperm. The first one involves Ca^{2+} efflux through the Plasma Membrane Ca^{2+} ATPase (PMCA), and the Na^+/Ca^{2+}-exchanger (NCX). PMCA, localized in the principal piece of the flagellum, is crucial for sperm function since its elimination affects sperm motility (Schuh *et al.*, 2004) and sperm from *PMCA4b* KO mice are deficient in hyperactivated motility and therefore sterile (Okunade *et al.*, 2004). Mitochondrial abnormalities found in PMCA4-deficient spermatozoa (Okunade *et al.*, 2004) suggest Ca^{2+} overload due to defective Ca^{2+} extrusion. NCX is present in the plasma membrane of mammalian spermatozoa (Babcock and Pfeiffer, 1987) and is thought to play a crucial role in the regulation of Ca^{2+} homeostasis (Reddy *et al.*,

2001; Su and Vacquier, 2002). Pharmacological inhibition of NCX leads to a rise in $[Ca^{2+}]_i$ and significant inhibition of human sperm motility (Krasznai *et al.*, 2006). The second Ca^{2+} transport system is related to Ca^{2+} influx and involves mainly the sperm-specific Ca^{2+} channel called CatSper (Ren *et al.*, 2001; Quill *et al.*, 2003; Jin *et al.*, 2007; Qi *et al.*, 2007). CatSper is composed by at least 7 subunits, and KO of any of them results in degradation of all other subunits (Qi *et al.*, 2007). Studies using these mice revealed that CatSper is essential for hyperactivation and fertilization. In addition, in contrast to what occurs in wild-type sperm, CatSper1 KO undergoes PKA activation and an increase in tyrosine phosphorylation even in nominal zero Ca^{2+} media suggesting that CatSper transports the Ca^{2+} involved in the regulation of PKA/cAMP-dependent pathway required for sperm capacitation (Navarrete *et al.*, 2015). Despite other Ca^{2+} plasma membrane channels being described, such as voltage-gated Ca^{2+} channels (Wennemuth *et al.*, 2000; Treviño *et al.*, 2004), cyclic-nucleotide gated Ca^{2+} channels (Wiesner *et al.*, 1998) and canonical transient receptor potential (TRPC) channels (Treviño *et al.*, 2001), only CatSper (Ren *et al.*, 2001; Quill *et al.*, 2003) was shown to be required for male fertility. In the present study, the increase in $[Ca^{2+}]_i$ that occurs under capacitating conditions is absent either in the presence of CatSper inhibitors or in CatSper KO sperm. Surprisingly, a small increase in $[Ca^{2+}]_i$ was observed in CatSper KO sperm incubated under capacitating conditions, suggesting that other transporters/channels may be contributing to increase Ca^{2+} levels during capacitation. This is in agreement with the fact that we have observed a small increase in intracellular Ca^{2+} in sperm incubated under non-capacitating conditions. Currently, no other Ca^{2+} currents have been recorded in mature mouse sperm and this possibility requires further experimentation.

Recent groundbreaking work from Chung and coworkers using super-resolution microscopy (STORM) showed that CatSper distributes longitudinally along four backbone lines localized in the plasma membrane close to the fibrous sheath (Chung *et al.*, 2014). Together with CatSper, other signaling molecules display a similar spatial distribution along the principal piece, which reveals a complex organization of signaling pathways in the sperm flagellum. In addition, a variation in subflagellar localization of CatSper domains in capacitated sperm has been described by 3D STORM (Chung *et al.*, 2014). Only 30% of sperm, approximately, presented a quadrilateral CatSper1 domain organization and were the ones able to display hyperactivated motility and tyrosine phosphorylation (Chung *et al.*, 2014). This is consistent with the observation made by different groups that only a subpopulation of sperm achieved hyperactivation upon capacitation (Kulanand and Shivaji, 2001; Buffone *et al.*, 2009; Goodson *et al.*, 2011). In agreement with this evidence, we could determine that approximately 40% of sperm increases $[Ca^{2+}]_i$ as a result of capacitation.

Despite the advantage previously described in using flow cytometry, this approach does not provide information about subcellular localization of $[Ca^{2+}]_i$ changes, as it does live single cell imaging. Both methods can be combined in a novel and powerful technique to study intracellular events such as the image-based flow cytometry, which allows the analysis of a large number of cells and provides subcellular localization of the labeling. This approach has recently been used with human sperm (Matamoros-Volante *et al.*, 2017) and may contribute to solve the controversy in the field regarding the origin of the $[Ca^{2+}]_i$ increase in mammalian sperm, and therefore the nature of the channels involved.

In humans but not mice, progesterone (Lishko, Botchkina and Kirichok, 2011; Strünker *et al.*, 2011) and other steroids activate CatSper via binding to the serine hydrolase ABHD2 (*α/β hydrolase domain–containing protein 2*) (Miller *et al.*, 2016; Mannowetz, Miller and Lishko, 2017). In both species, CatSper is weakly voltage dependent but its currents are strongly augmented by intracellular alkalinization perhaps related to the remarkable abundance of histidine residues in its amino terminus (Kirichok, Navarro and Clapham, 2006). In addition, previous reports have shown that BSA induces $[Ca^{2+}]_i$ influx through CatSper channel activation (Xia and Ren, 2009), because this response is absent in CatSper1 KO sperm. We could determine that the capacitation-associated $[Ca^{2+}]_i$ increase is related to both principal components of capacitating media, HCO_3^- and BSA. HCO_3^- stimulates a rise in $[Ca^{2+}]_i$ very rapidly as observed in live single cell analysis. High concentrations of HCO_3^- that sperm encounters in the female genital tract could trigger an initial change in the pHi and activation of SLO3 channels (Santi *et al.*, 2010); the resulting membrane hyperpolarization raises pHi, probably through a voltage-sensitive NHE mechanism (Chávez *et al.*, 2014). This intracellular alkalization activates CatSper channel, which results in a very rapid $[Ca^{2+}]_i$ increase.

Our work showed that only a subpopulation of sperm increases $[Ca^{2+}]_i$ during capacitation. This rise might be associated with the fact that only a fraction of the population undergoes capacitation. Nowadays cell sorting technology is available and may be used to separate capacitated sperm and therefore, improve *in vitro* fertilization success rates. Moreover, by using the appropriate fluorescent probes, flow cytometry can be also used to simultaneously monitor different capacitation-related events (e.g. Na^+, pH, Ca^{2+}, membrane potential, acrosomal exocytosis, etc) in the same sperm population. In this regard, it is well established that Ca^{2+} is essential for acrosomal exocytosis and evidence provided by different groups suggest that a combination of both, cell surface channels and intracellular stores contribute to initiation of acrosome reaction (De Blas *et al.*, 2002; Herrick *et al.*, 2005; Cohen *et al.*, 2014). Our results will help to understand how Ca^{2+} is modulated during capacitation in preparation for acrosomal exocytosis. Ideally, an image-based flow cytometry (Matamoros-Volante *et al.*, 2017) experiment where Ca^{2+} and acrosomal exocytosis is simultaneously monitored would help to understand this complex regulation.

Given the fact that most studies in mammalian sperm capacitation have been conducted *in vitro*, it is still unclear which are the *in vivo* mechanisms involved in this process. In this regard, we and others have started analyzing *in vivo* capacitation using mtDsRed2/Acr-EGFP mouse to monitor the status of the acrosome in live, motile sperm during their transit through the oviduct (Hino *et al.*, 2016; La Spina *et al.*, 2016). We envision that these approaches using new transgenic technologies developed to monitor intracellular events will reveal how capacitation occurs *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: This work was supported by the National Institute of Health (R01TW008662 to MGB), *Agencia Nacional de Promoción Científica y Tecnológica* (PICT 2015-2294 to MGB and PICT 2014-2702 to DK) and the Eunice Kennedy Shriver National Institute of Child Health and Human Development NIH (RO1 HD38082 to PEV).

We would like to thank Dr. Gabriel Rabinovich and Dr. Mariana Salatino for their assistance in the flow cytometry experiments. We also thank Dr. Maria Gracia Gervasi and Dr. Ana Maria Salicioni for their technical assistance, as well as Professor Ricardo Bello for his assistance in statistical analysis. We thank as well Rodrigo Vena for his technical assistance in the use of confocal microscopy. We also would like to thank Rene Baron, Fortabat and Williams foundations. We thank the Government of Extremadura (Spain) and the *Fondo Social Europeo* (PO14005) for the fellowship to D.M.H. and the *Consejo Nacional de Investigaciones Científicas Y Técnicas* for fellowships to G.M.L, T.D.M, C.R, L.C.P.M and A.R. The authors do not have conflict of interest to declare.

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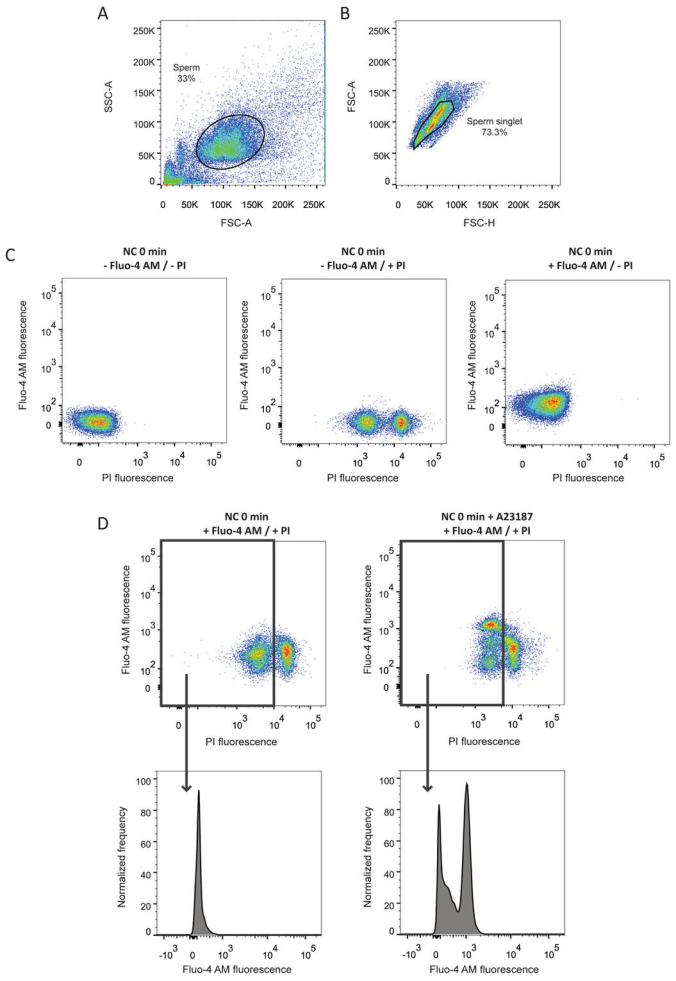

Figure 1. Parameters settings for $[Ca^{2+}]_i$ analysis by flow cytometry

Ca^{2+} was measured in mouse sperm in TYH medium using 1 μM Fluo-4 AM. **A)** Representative two-dimensional dot plot side-scatter area (SSC-A) vs. forward-scatter area (FSC-A) analysis of sperm collected after swim-out (NC 0 min) where the population of sperm is defined for each experiment (n=25). **B)** Representative two-dimensional dot plot FSC-A vs. forward-scatter height (FSC-H) analysis for doublet exclusion. Events deviating from the diagonal are doublets (n=25). **C)** Flow cytometry compensation controls performed in all experiments (n=25): Representative two-dimensional fluorescence dot plot Fluo-4 AM vs. PI analysis of sperm collected after swim-out (NC 0 min) without any staining (-Fluo-4 AM / -PI), with only PI staining (-Fluo-4 AM / +PI), or only Fluo-4 AM staining (+Fluo-4 AM / -PI). **D)** Fluo-4 AM vs. PI two-dimensional fluorescence dot plot analysis of sperm collected after swim-out (NC 0 min) incubated with or without Ca^{2+} ionophore A23187. These two-dimensional dot plots were used to distinguish between sperm with low (live) and high (dead) PI staining. Histogram analysis depicting normalized frequency of sperm and Fluo-4 AM fluorescence were performed in live sperm populations. Representative images of 4 independent experiments are shown.

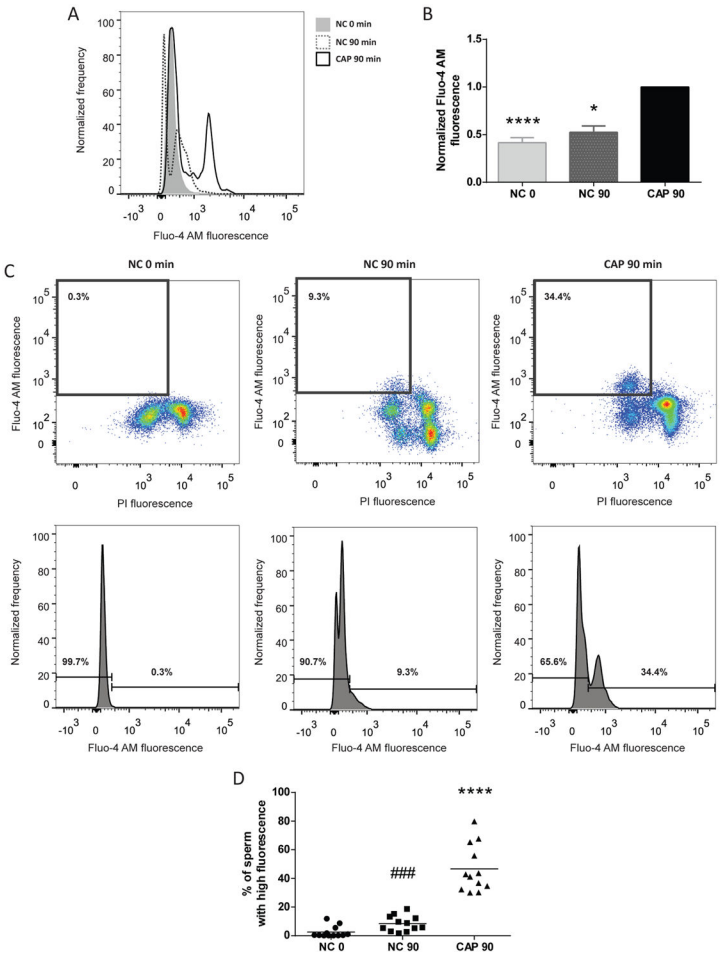

Figure 2. Intracellular Ca^{2+} levels increased during capacitation

A) Sperm collected after swim-out (NC 0 min) or incubated for 90 min either under non-capacitating conditions (NC) or capacitating conditions (CAP) were analyzed. Representative histograms of normalized frequency vs. Fluo-4 AM fluorescence of non-PI stained sperm are shown. **B)** Normalized MFI of Fluo-4 AM compared to the control condition (CAP 90 min). Values represent the mean ± SEM of 12 independent experiments. **** $p<0.0001$ represents statistical significance between control (CAP 90 min) vs. NC 0 min; * $p<0.05$ represents statistical significance between CAP 90 min vs. NC 90 min. Non-parametric Friedman test was performed in combination with Dunn's multiple comparisons test. **C)** Representative two-dimensional Fluo-4 AM vs. PI fluorescence dot plot and their corresponding histograms of normalized frequency vs. Fluo-4 AM fluorescence of non-PI stained sperm are shown. We identified the responsive population with high Fluo-4 AM fluorescence levels from the capacitating condition (CAP 90 min) and we established the percentage of sperm that responds increasing the $[Ca^{2+}]_i$. **D)** Percentage of sperm that increased Fluo-4 AM fluorescence after being incubated for 90 min under non-capacitating (NC 90 min) and capacitating conditions (CAP 90 min) in comparison with sperm collected after swim-out (NC 0 min). Values represent the percentage of sperm of 12 independent experiments. **** $p<0.0001$ represents statistical significance between control (CAP 90

min) vs. NC 0 and NC 90 min conditions; ### $p<0.001$ represents statistical significance between NC 90 min vs. NC 0 min condition. One-way ANOVA for matched data was performed, with Tukey's multiple comparisons test.

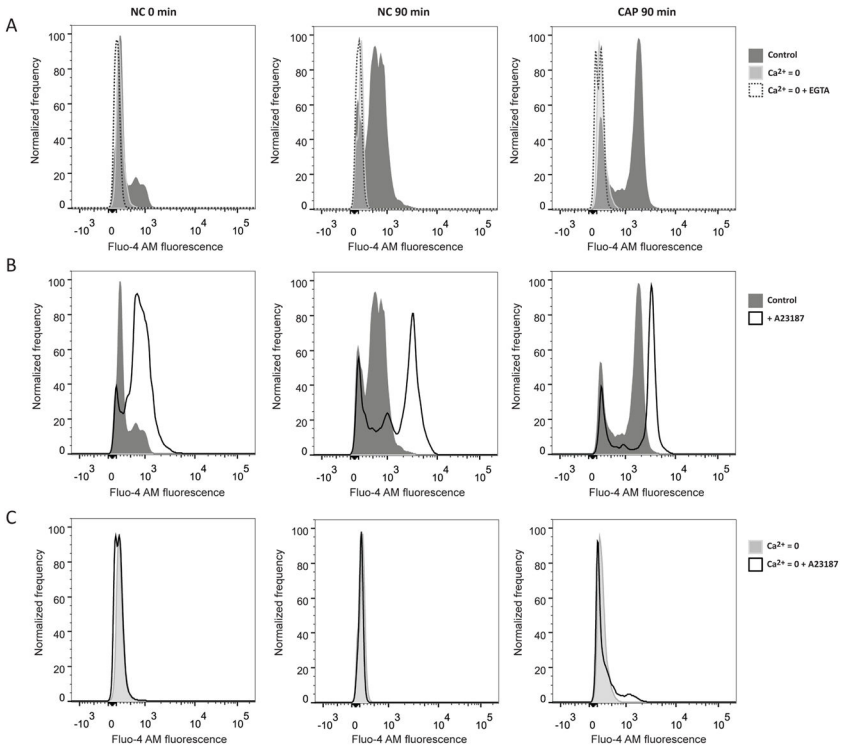

Figure 3. Capacitation-associated $[Ca^{2+}]_i$ increase depends on the presence of Ca^{2+} in the incubation medium

Cauda epididymal sperm were recovered by swim-out (NC 0 min) in media with or without Ca^{2+} (nominal zero Ca^{2+}, indicated by Ca^{2+}=0). Sperm were incubated for 90 min under non-capacitating (NC) and capacitating conditions (CAP) in media with or without Ca^{2+} (in the presence of EGTA 1 mM or not). Just before recording, in some cases, 10 μM of A23187 was added. Histograms of normalized frequency vs. Fluo-4 AM fluorescence of non-PI stained sperm are shown, which are representative of 4 independent experiments. **A)** Sperm collected after swim-out (NC 0 min). **B)** Sperm incubated for 90 min under non-capacitating conditions (NC 90 min). **C)** Sperm incubated for 90 min under capacitating conditions (CAP 90 min).

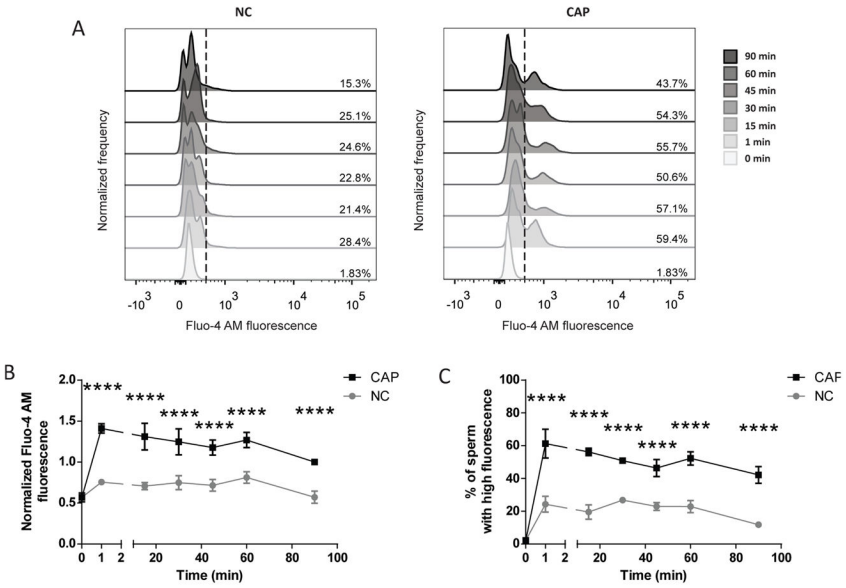

Figure 4. A rise in $[Ca^{2+}]_i$ occurs after 1 min of incubation under capacitating conditions

A) Intracellular $[Ca^{2+}]$ was measured by flow cytometry in sperm collected after swim-out (0 min) and incubated under non-capacitating and capacitating conditions for different time periods (1–90 min). Histograms of normalized frequency vs. Fluo-4 AM fluorescence of non-PI stained sperm, with the corresponding percentage of sperm that increased Fluo-4 AM fluorescence, are shown. Histograms are representative of at least 4 independent experiments. **B)** Normalized MFI, compared to the control condition (CAP 90 min), of Fluo-4 AM. Values represent the mean ± SEM of at least 4 independent experiments. **** $p<0.0001$ represents statistical significance between control (CAP 90 min) vs. time-matched NC condition. Two-way ANOVA with repeated measures and Sidak's multiple comparisons test was performed. **C)** Percentage of sperm that increased Fluo-4 AM fluorescence after being incubated (1–90 min) under non-capacitating (NC) and capacitating conditions (CAP). Values represent the mean ± SEM of at least 4 independent experiments. **** $p<0.0001$ represents statistical significance between control (CAP 90 min) vs. time-matched NC condition. Two-way ANOVA with repeated measures and Sidak's multiple comparisons test was performed.

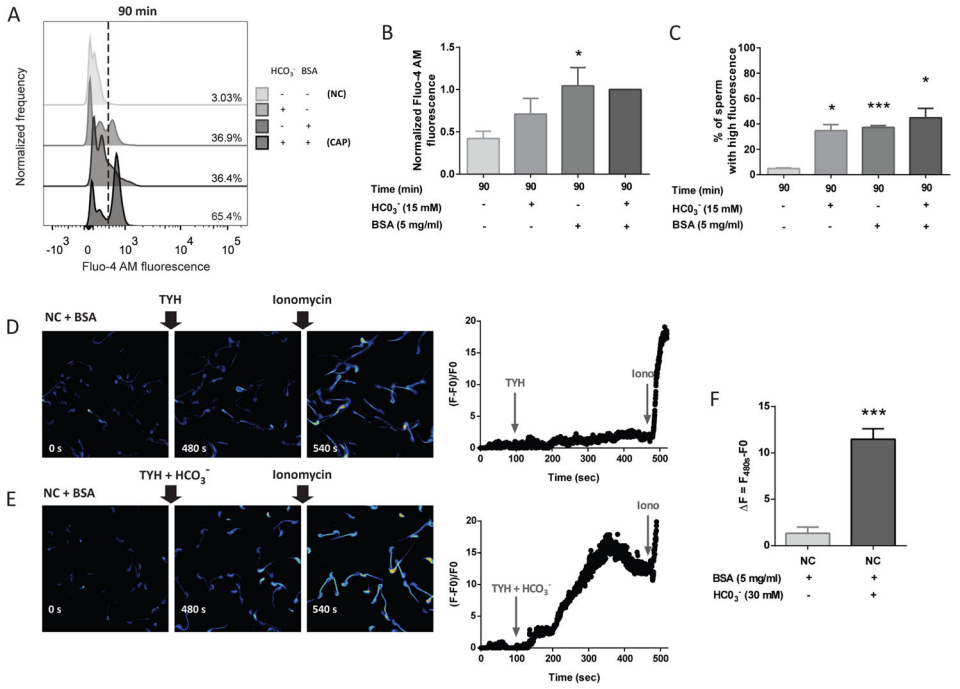

Figure 5. Either HCO_3^- or BSA caused independently an increase in Ca^{2+} response, being the effect of BSA significantly greater

Assessment of the effects of HCO_3^- and BSA on the capacitation induced $[Ca^{2+}]_i$ increase. **A)** Sperm were swum-out in non-capacitating medium (NC) and then incubated for 90 min in TYH medium in the presence or absence of different compounds: HCO_3^- (15 mM) and BSA (5 mg/ml). Histograms, representative of 4 independent experiments, of normalized frequency vs. Fluo-4 AM fluorescence of non-PI stained sperm are shown, with the corresponding percentage of sperm that increased Fluo-4 AM fluorescence. **B)** Normalized MFI, compared to the control condition (CAP), of Fluo-4 AM. Values represent the mean ± SEM of 4 independent experiments. * $p<0.05$ represents statistical significance between BSA (-HCO_3^-, +BSA, +Ca^{2+}) vs. NC condition (-HCO_3^-, -BSA, +Ca^{2+}). Non-parametric Friedman test was performed in combination with Dunn's multiple comparisons test. **C)** Percentage of sperm that increased Fluo-4 AM fluorescence after being incubated for 90 min in the different conditions. Values represent the mean ± SEM of 4 independent experiments. *** $p<0.001$, * $p<0.05$ represents statistical significance vs. NC condition (-HCO_3^-, -BSA, +Ca^{2+}). One-way ANOVA for matched data was performed, with Tukey's multiple comparisons test. **HCO_3^- addition promoted a rapid $[Ca^{2+}]_i$ increase in sperm. D–F)** Representative fluorescence images of 4 independent experiments, corresponding to $[Ca^{2+}]_i$ responses obtained before and after TYH medium or TYH+HCO_3^- addition, are shown. At the end 10 μM Ionomycin (Iono) was added as a viability control. Right traces show representative single cell $[Ca^{2+}]_i$ recordings obtained during each experiment. Arrows indicate additions of TYH or TYH+HCO_3^- and Ionomycin. **D)** Sperm exposed to vehicle (TYH medium). **E)** Addition of TYH with 30 mM HCO_3^-. **F)** Intracellular $[Ca^{2+}]$ increase under the indicated condition. ΔF = Fluorescence just before Ionomycin addition (480 s) – F0. Values represent the mean ± SEM of 4 independent experiments. *** $p<0.001$ represents

statistical significance between TYH+HCO_3^- vs. control condition (TYH addition). Paired t-test was performed.

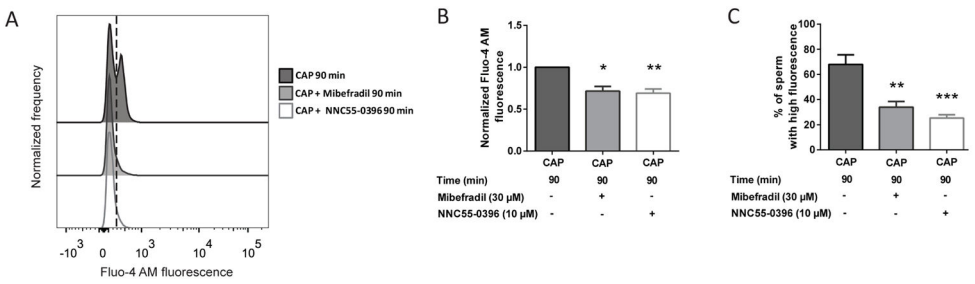

Figure 6. CatSper inhibition blocked the rise in $[Ca^{2+}]_i$ that occurs during capacitation

A) Sperm incubated for 90 min under capacitating (CAP) conditions in the absence or presence of CatSper inhibitors (Mibefradil and NNC55-0396), were analyzed. Representative histograms of normalized frequency vs. Fluo-4 AM fluorescence of non-PI stained sperm, with the corresponding percentage of sperm that increased Fluo-4 AM fluorescence, are shown. **B)** Normalized MFI, compared to the control condition (CAP without CatSper inhibitor), of Fluo-4 AM. Values represent the mean ± SEM of 8 independent experiments. ** $p<0.01$, * $p<0.05$ represents statistical significance vs. CAP condition without CatSper inhibitor. Non-parametric Friedman test was performed in combination with Dunn's multiple comparisons test. **C)** Percentage of sperm that increased Fluo-4 AM fluorescence after being incubated for 90 min in the different conditions. Values represent the mean ± SEM of 8 independent experiments. *** $p<0.001$, ** $p<0.01$ represents statistical significance vs. (CAP without CatSper inhibitor). One-way ANOVA for matched data with Dunnett's multiple comparisons test was performed.

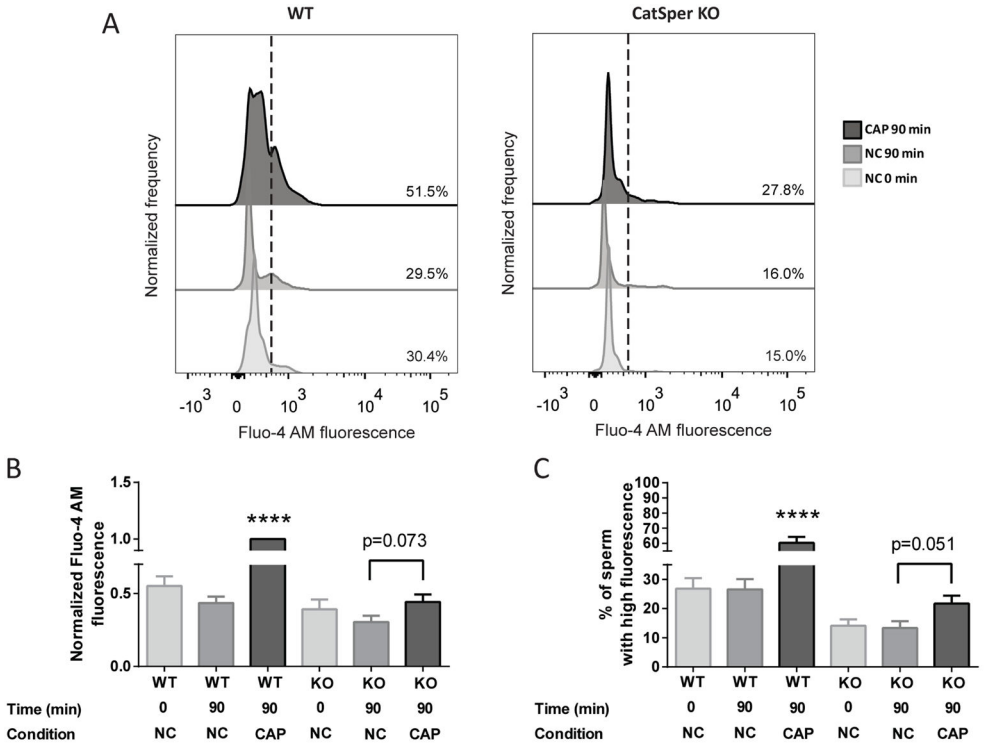

Figure 7. Functional CatSper channels are required for the capacitation associated $[Ca^{2+}]_i$ increase to occur

A) CatSper KO and wild-type mice sperm collected after swim-out (NC 0 min) or incubated for 90 min either under non-capacitating conditions (NC) or capacitating conditions (CAP) were analyzed. Representative histograms of normalized frequency vs. Fluo-4 AM fluorescence of non-PI stained sperm, with the corresponding percentage of sperm that increased Fluo-4 AM fluorescence, are shown. **B)** Normalized MFI, compared to the control condition (wild-type CAP), of Fluo-4 AM. Values represent the mean ± SEM of 5 independent experiments. Two-way ANOVA showed a significant interaction (genotype x condition), $P_{interaction}<0.001$. **** $p<0.0001$ represents statistical significance between control (wild-type CAP condition) vs. all other conditions. Sidak's multiple comparisons test was performed. To analyzed the differences between CatSper KO NC and CAP conditions, paired t-test was performed (p=0.073). **C)** Percentage of sperm that increased Fluo-4 AM fluorescence after being incubated in the different conditions. Values represent the mean ± SEM of 5 independent experiments. Two-way ANOVA showed a significant interaction (genotype x condition), $P_{interaction}<0.001$. **** $p<0.0001$ represents statistical significance between control (wild-type CAP condition) vs. all other conditions. Sidak's multiple comparisons test was performed. In order to determine the differences between CatSper KO NC and CAP conditions, paired t-test was performed (p=0.051).