Replacement of calcium for strontium in hamster sperm incubation media: effect on sperm function

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ABSTRACT: Calcium (Ca²⁺) is an absolute requirement for a decisive sperm function event: the acrosome reaction (AR). Physiologically, sperm capacitation is a prerequisite for this specialized exocytosis and both events are intimately related. In an effort to separate capacitation from AR, we have been using a modified sperm incubation medium where Ca²⁺ is replaced by Strontium (Sr²⁺). The aim of this report is to analyze with more detail the difference between sperm incubated with Ca²⁺ or Sr²⁺ in several events. We found that sperm undergo the capacitation-related changes in the chlortetracycline (CTC) pattern and tyrosine phosphorylation, and also bind to the zona pellucida (ZP) when using Sr²⁺-instead of Ca²⁺-containing media. However, the spontaneous AR typical of hamster sperm does not take place in Sr²⁺-medium, even if sperm are previously capacitated with Ca²⁺. Nevertheless, Sr²⁺ was able to sustain AR when cells were treated with thapsigargin or depolarized with K⁺ in Na⁺-depleted medium. Considering that the absence of Na⁺ increased spontaneous AR in Sr²⁺-medium, we tested whether Na⁺-transport systems could be involved in the inability of Sr²⁺-incubated sperm to undergo AR. We found that when sperm incubated in Sr²⁺-medium are treated with amiloride to inhibit epithelial Na⁺-transport systems, they are able to undergo spontaneous AR. The same result was obtained when analyzing AR on the ZP. On the contrary, addition of ouabain (a Na⁺/K⁺-ATPase inhibitor) or DIDS (a Na⁺/HCO₃⁻ co-transporter inhibitor) showed no effect. These results suggest that, differing from what happens in Ca²⁺-incubated sperm, cells incubated in Sr²⁺-modified medium would have an active ENaC.

Key words: acrosome reaction / calcium / capacitation / spermatozoa

Introduction

Mammalian sperm must complete an ultimate differentiation process within the female tract to become fertile. The group of biochemical and functional changes associated with this process is known as capacitation (Austin, 1951; Chang, 1951). Although the complete mechanism underlying this process has not been elucidated, some events are related to it, e.g. cholesterol efflux (Davis et al., 1980; Cross, 1998); modification of the intracellular concentration of ions (Fraser, 1995); increase in the degree of tyrosine phosphorylation of several proteins (Visconti et al., 1995); hyperpolarization of the sperm plasma membrane (Zeng et al., 1995).

By the time sperm reach the oocyte, a series of coordinated events take place: sperm binds to the oocyte zona pellucida (ZP), inducing an exocytotic event known as acrosome reaction (AR), which allows sperm to penetrate the ZP. All the structural and functional changes that take place during capacitation are assumed to lead to the exposure, assembly and/or activation of the mechanisms involved in oocyte recognition. Simultaneously or as a consequence of these structural changes, signaling pathways become ready for sperm to undergo the AR after binding to the ZP.

Although the exocytosis is ultimately triggered by a massive increase in intracellular Ca²⁺, there is a set of sequential steps preceding this final event. Compared with somatic cells, sperm are depolarized; e.g. their membrane potential is more positive (Zeng et al., 1995). As a consequence of capacitation, sperm plasma membrane hyperpolarizes and voltage-dependent calcium channels become ready to be activated by its ligand (Zeng et al., 1995; Arnoult et al., 1999). Subsequently, binding of sperm receptors to the ZP induces plasma membrane depolarization and opening of these channels. A small increase of cytoplasmic Ca²⁺ triggers a signal transduction event that opens the acrosomal store (Florman, 1994; Zeng et al., 1995). The consequent
activation of store-operated Ca\(^{2+}\) channels (O’Toole et al., 2000; De Blas et al., 2002) and the massive Ca\(^{2+}\) influx allows the fusion of the plasma and the acrosomal membranes (Breitbart, 2002; Darzson et al., 2005).

Previous results from our laboratory suggest that the Ca\(^{2+}\) contained in sperm incubation media could be replaced by Sr\(^{2+}\) in order to complete both capacitation and binding to the ZP. However, AR could not take place in Sr\(^{2+}\)-modified medium (Marin-Briggiler et al., 1999; Zitta et al., 2004). Considering these results, we hypothesized that Sr\(^{2+}\) was equivalent to Ca\(^{2+}\) in the events associated with capacitation and binding to the ZP, but not in the signaling pathway leading to AR. The aim of this study is to further analyze this possibility and examine similarities and differences between Ca\(^{2+}\) and Sr\(^{2+}\) throughout capacitation and AR. Accordingly, we will assess whether Sr\(^{2+}\) is able to support capacitation, evaluating several parameters associated with this process. Besides, different conditions to induce AR will be used in order to explore where along the signaling pathway Sr\(^{2+}\) differs from Ca\(^{2+}\). Finally, we analyze the participation of different ion transport systems in the functional differences between cells incubated in Ca\(^{2+}\)- or Sr\(^{2+}\)-containing media.

Materials and Methods

Materials

All reagents used were of highest purity or analytical grade and purchased from Sigma (St Louis, MO, USA), Fisher (Fairlawm, NJ, USA), Merck (Darmstadt, Germany) or J.T. Baker (Phillipsburg, NJ, USA). Fatty-acid-free bovine serum albumin (BSA) was from Serological Corporation (Kankakee, IL, USA); the pregnant mare’s serum gonadotropin (PMSG) used was Novormon (Sintex, Buenos Aires, Argentina).

Spermatozoa

Hamster sperm were obtained from adult male epididymides by swim-out in sperm washing medium [SWM: 72 mM NaCl, 1.5 mM KCl, 3.2 mM Na\(_2\)HPO\(_4\), 0.6 mM KH\(_2\)PO\(_4\), 0.5 mM CaCl\(_2\), 0.4 mM MgCl\(_2\), 150 mM sucrose and 1 mg/ml of polyvinyl alcohol (PVA)] and purification through a glass bead column as described previously (Bavister, 1989). Sperm were incubated under capacitating conditions using TL medium (37°C, 5% CO\(_2\) atmosphere). Aliquots of the purified sperm suspension (100,000 cells/ml) were mixed with one volume of CTM solution (750 μM CTC, 5 mM Cysteine in 20 mM Tris, 130 mM NaCl, pH 7.4; Ward and Storey, 1984; Fraser and Herod, 1990). Following 15 min incubation, cells were fixed with formaldehyde (4% final concentration) for 15 min. Fluorescence signal was observed using a Nikon Optiphot microscope with epifluorescent illumination (Howard filter, 330–380 nm; Saling and Storey, 1979). The proportion of cells displaying pattern B (fluorescence in the tail and acrosome and a dark post-equatorial region), associated with capacitated sperm, was quantified in cells incubated in TALP or TALP-Sr at the beginning and after 3 h of incubation.

Oocytes

Prepubertal female hamsters were superovulated by administration of 40 IU of PMSG, followed by 40 IU of hCG (Sigma, MO, USA) 50–65 h later. Oocytes were collected from the oviduct 15–17 h post-hCG and were freed of cumulus cells by treatment with 0.1% hyaluronidase for 5 min. Oocytes were then washed by pipetting through five droplets of TALP supplemented with 5 mM HEPES and maintained up to 2 months in 0.5 M (NH\(_4\))\(_2\)SO\(_4\), 0.75 M MgCl\(_2\), 0.2 mM ZnCl\(_2\) and 0.1 mg/ml of PVA, pH 7.4, at 4°C, until use (Boatman et al., 1988).

Binding assays

Salt-stored oocytes (10–15/droplet) were extensively washed by pipetting (1.5 h total time) through five droplets of TALP or TALP-Sr supplemented with 5 mM HEPES and placed in 50 μl droplets of fresh medium before use (Zitta et al., 2004). Oocytes were inseminated with sperm previously incubated under capacitating conditions for 3 h in the same medium (1 × 10\(^5\) sperm/ml). Following co-incubation for 45 min at 37°C under 5% CO\(_2\), oocytes were transferred to fresh droplets of the respective medium containing 1% sodium azide, washed by pipetting to remove unbound sperm and fixed in 0.1% formaldehyde for 5 min. The number of sperm bound per oocyte was determined by microscopic examination. When analyzing the ability of Sr\(^{2+}\) to sustain sperm capacitation and binding, ethylene glycol tetaacetic acid (EGTA) 0.1 mM was present during both sperm pre-incubation and sperm–oocytes co-incubation.

CＴC staining

An aliquot (45 μl) of the sperm suspension was mixed with one volume of CTC solution (750 μM CTC, 5 mM Cysteine in 20 mM Tris, 130 mM NaCl, pH = 7.4; Ward and Storey, 1984; Fraser and Herod, 1990). After a 5 min incubation, cells were fixed with formaldehyde (4% final concentration) for 15 min. Fluorescence signal was observed using a Nikon Optiphot microscope with epifluorescent illumination (Howard filter, 330–380 nm; Saling and Storey, 1979). The proportion of cells displaying pattern B (fluorescence in the tail and acrosome and a dark post-equatorial region), associated with capacitated sperm, was quantified in cells incubated in TALP or TALP-Sr at the beginning and after 3 h of incubation.

Electrophoresis and western blot

Total sperm extracts were obtained by cell suspension in non-reducing Laemmli buffer and boiling for 5 min. Before running, samples were supplemented with 5% β-mercaptoethanol. After electrophoresis on a 10% SDS-denaturing gel, proteins were electroblotted to PVDF membrane and blocked with 5% BSA. All the incubation and washing procedures were carried out with PBS supplemented with 0.1% Tween 20 (PBST). Membranes were blocked for 1 h at room temperature and then incubated with monoclonal anti-phosphotyrosine antibody (1:10,000, Upstate Biotechnology, NY, USA) overnight at 4°C. After washing three times for 5 min, peroxidase-conjugated secondary antibody (1:5000, Jackson Laboratories, CA, USA) was added and incubation carried out for 1 h at room temperature. The membranes were washed with PBST, and immune complexes located using ECL Plus (Amersham Pharmacia Biotech Inc., NJ, USA) and Kodak Biomax Light Films. Molecular weight standards were from BioRad (Precision Plus, Dual color, CA, USA).

Treatments

Ethylene glycol tetaacetic acid

Two different analysis involved chelation of divalent cations. In one of them, the goal was to eliminate any possible Ca\(^{2+}\) remnants in the
medium where CaCl₂ was replaced by SrCl₂. For that purpose, 0.1 mM EGTA was included in the Sr²⁺ medium used for both sperm incubation and sperm–oocytes co-incubation. In a different experimental set, we investigated whether the extracellular ion was required to support AR. In this case, 1.8 mM EGTA was added after 3 h of sperm incubation in order to chelate all the divalent cation before treatment (see below).

Thapsigargin (TG)
Cells incubated in TALP or TALP-Sr for 3 h were centrifuged at 600g for 3 min and resuspended in fresh Ca²⁺ or Sr²⁺ medium. To rule out any artifact, control cells were treated similarly but resuspended in the original medium. AR was evaluated 30 min after adding 3.75 µM TG (Llanos, 1998). As a control, an identical volume of vehicle (ethanol) was added to sperm. To check that extracellular ions were necessary for TG to induce AR, 1.8 mM EGTA was added to sperm just before TG.

Amiloride
To analyze the possible participation of epithelial Na⁺ channels (ENaC) in the event under study, sperm were incubated for 3 h under capacitating conditions and 1 µM Amiloride was then added to the incubation vessels. AR was evaluated 30 min later and at the end of the incubation.

Depolarization
Sperm were placed in a modified TALP lacking NaCl (TALP-N) with CaCl₂ or SrCl₂. Depolarization was induced by addition of 60 mM KCl (Florman et al., 1992) and AR measured 30 min later. To prevent the effect of different osmolarities, NaCl was replaced with an equivalent concentration (101 mM) of Choline chloride. This treatment was repeated using capacitated cells: sperm incubated for 3 h were centrifuged and resuspended in TALP or TALP-N and depolarized with KCl.

DIDS, ouabain
After sperm incubation in TALP-Sr for 3 h, wells were supplemented with 4,4′-diodothiocyanostilbene-2,2′-disulfonic (DIDS, 100 µM) or ouabain (1 µM), and AR evaluated 30 min later. To verify the efficacy of these agents, control wells containing sperm in Ca²⁺ medium were supplemented or not with the inhibitor at the beginning of the incubation.

AR on the ZP
In order to analyze the occurrence of AR on the ZP, cells were incubated according to the procedure previously described (Uto et al., 1988). Briefly, salt-stored hamster oocytes (10–15/droplet) were extensively washed by pipetting (1.5 h total time) through five droplets of medium and inseminated with sperm (500,000 cells/ml) previously incubated for 3 h in TALP or TALP-Sr. After a 5 min co-incubation at 37°C in 5% CO₂, cells were washed by pipetting in several drops of the respective medium and transferred to fresh droplets containing no sperm. Two hours later, oocytes were mounted and the acrosomal status of motile sperm bound to the ZP was determined (Zitta et al., 2004). Both sperm capacitation and co-incubation with the oocytes was carried out in the same medium, i.e. TALP or TALP-Sr. In order to assess the effect of amiloride on the ZP-induced AR, two different approaches were used: the agent was added during co-incubation or for the last 2 h after binding. Likewise, Ca²⁺ was added in a similar way to drops run in parallel.

Expression of results and statistical analysis
Results were expressed as the percentage of acrosome-reacted sperm (%AR) or the number of sperm bound per oocyte. In some cases, results were normalized to control values, which were considered as 100%. Graphics represent averages ± SEM of at least three different experiments. To assume normal distribution, percentages were converted to ratios and subjected to the arcsine square root transformation. Statistical analysis was performed using the Dunnett test to compare different treatments versus control, or the Bonferroni test for multiple comparisons. All statistical procedures were carried out with the aid of GraphPad software.

Results
Sr²⁺ versus Ca²⁺ in sperm capacitation
While studying the participation of carbohydrates in sperm–ZP interaction in hamsters, we noticed that sperm incubated in capacitating medium where Ca²⁺ was replaced by Sr²⁺ did not undergo the AR. However, they were ready to react once Ca²⁺ was provided at the time of exocytosis (Zitta et al., 2004). These results lead us to propose that Sr²⁺ was able to replace Ca²⁺ during capacitation. However, the possible contribution of Ca²⁺ traces from other components of the capacitation medium could not be ruled out. For this reason, we repeated these experiments, adding 0.1 mM EGTA to the Sr²⁺ medium.

The hamster is a unique species since, once capacitation is complete, most sperm undergo a VDCC-dependent spontaneous AR (Feng et al., 2007), which can be verified by light microscopy on motile cells (Yanagimachi and Phillips, 1984; Cross and Meizel, 1989). The proportion of reacted sperm is significantly increased after ~3 h of incubation, and almost all sperm are reacted 3 h later. At the end of the experiment, AR under control conditions (Ca²⁺ medium) reached maximal levels (90 ± 4%), while only a low proportion of cells were reacted when Sr²⁺ medium was used during the whole incubation (15 ± 3%, P < 0.001). However, when Ca²⁺ was added after the capacitation period had been completed in Sr²⁺ medium, AR reached levels similar to those of controls, irrespective of the presence of EGTA (90 ± 3 and 88 ± 3% with and without EGTA, respectively).

The ability of Sr²⁺ to sustain the acquisition of ZP-binding ability and binding itself was then re-analyzed. When both capacitation and binding assays were carried out in Sr²⁺ medium with or without 0.1 mM EGTA, no differences were detected when compared with control experiments carried out in Ca²⁺ medium. Results were expressed as percentage of control values and are shown in Fig. 1A.

In order to analyze the putative similarity between Ca²⁺ and Sr²⁺-incubated sperm, several variables associated with capacitation were investigated. Capacitation has been associated with a change in the CTC staining pattern (Ward and Storey, 1984). This was originally described in mice and later confirmed in all of the species tested. When CTC patterns in hamster sperm incubated in Ca²⁺ or Sr²⁺ medium were studied, the proportion of capacitated (pattern B) sperm was similar (Fig. 1B).

Protein tyrosine phosphorylation increases during capacitation and it is regularly used to verify that this process is taking place. In hamsters, the level of phosphorylation detected by western blot reaches its maximum around 90 min after the start of the incubation (Visconti et al., 1999). When the patterns of tyrosine phosphorylation in Ca²⁺ or Sr²⁺ incubated sperm were analyzed, no significant differences were observed (Fig. 1C).
In summary, no discrepancies were detected in Sr\textsuperscript{2+}-incubated sperm, although they diverge from Ca\textsuperscript{2+}-incubated cells in their ability to undergo spontaneous AR. Sustaining AR after depletion of the acrosomal store

AR is triggered by a massive entry of extracellular Ca\textsuperscript{2+} induced by depletion of the acrosomal store (De Blas et al., 2002). To explore whether Sr\textsuperscript{2+} could replace Ca\textsuperscript{2+} in the final massive entry, the previous requirement of store depletion was bypassed using TG. This Ca\textsuperscript{2+}-ATPase-pump inhibitor induces acrosomal Ca\textsuperscript{2+} release and the consequent influx of the extracellular ion required for AR (Meizel and Turner, 1993; Llanos, 1998; Herrick et al., 2005). Sperm were capacitated under control conditions (Ca\textsuperscript{2+} medium for 3 h) and then shifted to fresh Ca\textsuperscript{2+} or Sr\textsuperscript{2+}-containing medium. Resulting AR was determined 30 min later and at final incubation time. Sperm resuspended in Ca\textsuperscript{2+} medium were reacting after 30 min, while those placed in Sr\textsuperscript{2+} medium displayed basal levels of AR even at the end of the incubation (Fig. 2, Ca/Ca versus Ca/Sr at 3.5 and 6 h, respectively). These results indicate that even capacitated cells are unable to undergo spontaneous AR when Ca\textsuperscript{2+} in the medium is replaced by Sr\textsuperscript{2+}. However, addition of TG allowed sperm to undergo AR in Sr\textsuperscript{2+} medium, reaching levels similar to those of control at final incubation time (Ca/Sr + TG versus Ca/Ca, Fig. 2).

A similar procedure was repeated using sperm incubated in TALP-Sr for 3 h. A significantly increased rate of AR was observed 30 min after addition of TG, reaching levels similar to those moved to Ca\textsuperscript{2+} medium (Sr/Ca), or incubated under control conditions all the time (Ca) (Fig. 3). To verify that the increase in AR observed after TG addition was sustained by the extracellular ion, treatment with TG was preceded by the addition of 1.8 mM EGTA. Chelation of the extracellular ion, either Ca\textsuperscript{2+} or Sr\textsuperscript{2+}, blocked the increase in AR induced by TG (Fig. 3). Thimerosal was used as an alternative agent to induce the acrosomal store depletion (Herrick et al., 2005), and similar results were obtained (data not shown).

**Figure 1** Analysis of capacitation in sperm incubated in Ca\textsuperscript{2+}-free/Sr\textsuperscript{2+}-replaced medium. (A) Spontaneous AR and binding to ZP. Sperm were incubated in Sr\textsuperscript{2+}-modified medium alone (Sr) or supplemented with 0.1 mM EGTA (Sr + EGTA). After 3 h incubation, Ca\textsuperscript{2+} was added to some wells (Sr/Ca and Sr + EGTA/Ca). Spontaneous AR was analyzed at final incubation time. Aliquots of sperm incubated for 3 h in Sr\textsuperscript{2+}-containing medium with or without EGTA (Sr) were used in ZP-binding assays carried out in the same media used for sperm pre-incubation. Results were normalized against control assays developed in parallel using Ca\textsuperscript{2+}-medium (Ca). (B) CTC pattern. An aliquot of sperm suspended in Ca\textsuperscript{2+} (Ca) or Sr\textsuperscript{2+} (Sr)-containing medium were treated with CTC and the proportion of cells displaying the B pattern was quantified at the beginning (To) and once capacitation was completed (3 h). (C) Tyrosine phosphorylation. Aliquots of sperm incubated for 90 min in Sr\textsuperscript{2+} (Sr) or Ca\textsuperscript{2+} (Ca)-containing medium were analyzed by western blot with an anti-phosphotyrosine antibody. A sample of non-incubated sperm (To) was included to show the lack of signal before sperm incubation. *: P < 0.01 versus Sr/Ca, Sr + EGTA/Ca.
Taken together, these results suggest that once the acrosomal store is depleted, extracellular Sr$^{2+}$ can enter the cell and replace Ca$^{2+}$ in the final steps of the exocytotic process.

**Sustaining AR after depolarization**

Once the ability of Sr$^{2+}$ to replace Ca$^{2+}$ in the massive final entry was verified, we focused on the previous event in the Ca$^{2+}$-signaling cascade: opening of the acrosomal store. Considering that this event is triggered by the influx of extracellular divalent cation, we analyzed the ability of Sr$^{2+}$ to open the store forcing its entry by sperm depolarization. For that purpose, sperm need to be placed in a Na$^{+}$-free medium and then depolarized by the addition of K$^+$ (Babcock and Pfeiffer, 1987; Florman et al., 1992). Depolarization induced a similar level of AR in both media (Fig. 4). Nevertheless, the ratio of reacted cells was lower than the one usually obtained at final incubation time. Considering that voltage-dependent Ca$^{2+}$-channels in non-capacitated sperm are unavailable for opening, the same procedure was repeated using cells previously incubated under capacitating conditions. After being incubated for 3 h in Ca$^{2+}$-containing media, sperm were centrifuged and resuspended in fresh medium with or without Na$^{+}$. Again, depolarization of cells induced similar levels of AR in either Ca$^{2+}$- or Sr$^{2+}$-containing media (Fig. 5). Moreover, the resulting AR levels parallel those obtained in sperm incubated in Ca$^{2+}$ medium for 6 h. This result suggests that once Sr$^{2+}$ entered the cell, it induced the acrosomal store opening.

Interestingly, we observed that the sole resuspension of Sr$^{2+}$-incubated sperm in Na$^{+}$-free medium (i.e. without the addition of K$^+$) induced a significant increase in AR (see –Na bar in Fig. 5).

Figure 2 Analysis of Sr$^{2+}$ ability to sustain AR. Cells were incubated in Ca$^{2+}$-containing medium to allow capacitation. After 3 h, they were centrifuged and resuspended in Sr$^{2+}$-modified medium alone (Ca/Sr), supplemented with TG (Ca/Sr + TG), or its vehicle (Ca/Sr + SV). Spontaneous AR was evaluated 30 min later (3.5 h) and at final incubation time (6 h). Control wells using cells resuspended in Ca$^{2+}$-medium were run in parallel (Ca/Ca). *: $P < 0.05$ versus Ca/Ca and Ca/Sr + TG; #: $P < 0.001$ versus Ca/Ca at 3/5 h, Ca/Sr, Ca/Sr + SV.

Figure 3 AR in sperm incubated in Sr$^{2+}$-containing medium. Cells were incubated in Sr$^{2+}$-containing medium for 3 h, centrifuged and resuspended in the same medium alone (Sr/Sr) or supplemented with TG (Sr/Sr + TG). Alternatively, cells were resuspended in Ca$^{2+}$ medium (Sr/Ca). To verify that AR was being sustained by the extracellular Sr$^{2+}$, 1.8 mM EGTA was added before TG (Sr/Sr + EGTA + TG). Cells incubated in Ca$^{2+}$ medium alone (Ca/Ca), supplemented with TG (Ca/Ca + TG), or EGTA and TG (Ca/Ca + EGTA + TG) were used as controls. AR was quantified 30 min after addition of TG. *: $P < 0.001$ versus Sr/Sr, Sr/Sr + EGTA + TG; #: $P < 0.01$ versus Ca/Ca and Ca/Ca + EGTA + TG.
This result suggests that the presence of Na$^+$ is related to the absence of AR when sperm are incubated in Sr$^{2+}$ medium.

**Sperm incubated in Sr$^{2+}$ medium and Na$^+$ transport systems**

It has been proposed that the ENaC is functional in resting sperm, but turns off during capacitation, thus regulating the sperm membrane potential (Hernandez-Gonzalez et al., 2006). To analyze whether this Na$^+$-transport mechanism was related to the lack of AR in Sr$^{2+}$-incubated sperm, we used 1 $\mu$M amiloride, which specifically inhibits ENaC at this concentration (Teiwes and Toto, 2007). When amiloride was added to sperm incubated under capacitating conditions in Sr$^{2+}$ medium for 3 h, AR reached levels similar to those of controls (Fig. 6). This effect was observed 30 min after the addition of amiloride, and maintained until the end of the incubation. When Ca$^{2+}$-incubated sperm were treated with amiloride, no effect was observed, in agreement with the absence of an active ENaC.

After this specific difference related to spontaneous AR in sperm incubated in Ca$^{2+}$ or Sr$^{2+}$ was found, we decided to verify this effect in a more physiological condition: AR on the ZP. In agreement with our previous report (Zitta et al., 2004), the use of Ca$^{2+}$ or Sr$^{2+}$ during the whole experiment produced maximal and minimal AR values, respectively (Fig. 7). When Sr$^{2+}$-incubated sperm were co-incubated with oocytes in this same medium and in the presence of amiloride, AR reached values similar to controls (Sr$^+$ + Am versus Ca$^+$). The same result was obtained when amiloride was replaced by Ca$^{2+}$ (Sr$^+$ + Ca$^+$). These results validate those obtained when spontaneous AR was determined.

In order to assess whether the signaling cascade interrupted in a Sr$^{2+}$-containing medium could be resumed, amiloride was added to ZP-bound sperm. In this case, AR could not occur (Sr$^+$ + Am bar, Fig. 7), suggesting that the initial step of the pathway must be ready at the time sperm–ZP are binding. A similar result was obtained when Ca$^+$ was added after sperm–ZP binding in Sr$^{2+}$ medium (Sr$^+$ + Ca bar, Fig. 7).

Another Na$^+$-transport system that has been recently related to the capacitation-associated change in the membrane potential is the Na$^+$/HCO$_3^-$ co-transporter, blocked by 4,4-diidothiocyanatotribenene-2,2′-disulfonic acid (DIDS) (Demarco et al., 2003). To examine whether the Na$^+$/HCO$_3^-$ co-transporter was involved in the event under study, the effect of DIDS was tested. Sperm incubated in Sr$^{2+}$-medium for 3 h were treated with this inhibitor, and AR evaluated 30 min later. The rate of AR in Sr$^{2+}$-medium was not modified by DIDS (Fig. 8). As a control, this inhibitor was added at the beginning of sperm incubation in Ca$^{2+}$ medium, and spontaneous AR was accordingly blocked (Visconti et al., 1999). These results suggest that the absence of AR in Sr$^{2+}$-modified medium would not be related to the Na$^+$/HCO$_3^-$ co-transporter and/or any anion transport system sensitive to DIDS.

The involvement of Na$^+$/K$^+$-ATPase in capacitation was originally raised by Mrsny and Meizel (1981). Besides, this pump has been implicated in the sperm membrane potential changes associated with the presence or absence of extracellular Ca$^{2+}$ (Espinosa and Darszon, 1995; Gonzalez-Martinez, 2003; Torres-Flores et al., 2008). In order to analyze whether Na$^+$/K$^+$-ATPase was related to the absence of AR in Sr$^{2+}$-medium incubated sperm, we studied the effect of ouabain, a specific inhibitor of this pump. When sperm incubated for 3 h in Sr$^{2+}$-medium were treated with ouabain, no changes in AR were observed (Fig. 9). As a control, this inhibitor was added at the beginning of sperm incubation in Ca$^{2+}$ medium and spontaneous AR was blocked, in agreement with the previous reports (Mrsny and Meizel, 1981; Mrsny et al., 1984). These results indicate that Na$^+$/K$^+$-ATPase would not be responsible for the Na$^+$-related absence of AR in Sr$^{2+}$ medium.

**Discussion**

Since the description of sperm capacitation, and especially during the last two decades, a great deal of information about how sperm function is regulated has been uncovered. However, several questions...
remain to be answered. Some require particular experimental conditions allowing the analysis of different but intertwined events. This was the purpose of our initial study on Ca\textsuperscript{2+} replacement in sperm incubation media, which subsequently led us to further analyze the molecular basis of this system.

In a previous study, we analyzed the substitution of Ca\textsuperscript{2+} by Sr\textsuperscript{2+} in the incubation medium (Zitta et al., 2004). However, to verify that Sr\textsuperscript{2+} and not Ca\textsuperscript{2+} remnants were sustaining the capacitation-associated events, we included 0.1 mM EGTA to the Sr\textsuperscript{2+} medium to chelate any putative Ca\textsuperscript{2+} present during the incubation. Under this condition, ZP binding was similar to control in Ca\textsuperscript{2+} medium, and spontaneous AR also took place when Ca\textsuperscript{2+} was provided after the time assumed to be necessary for capacitation in hamsters. These results suggest that events taking place during sperm incubation in Sr\textsuperscript{2+} medium were sustained by this ion and not by Ca\textsuperscript{2+} traces.

CTC is commonly used as a probe of membrane-bound Ca\textsuperscript{2+} (Caswell and Hutchison, 1971; Saling and Storey, 1979). Sperm capacitation is associated with a specific CTC staining pattern (Ward and Storey, 1984). Since this dye also binds Sr\textsuperscript{2+} (Heffner et al., 1980), we could use sperm displaying the B pattern of CTC staining to follow capacitation in media containing any of the divalent cations. The rate of sperm with a B pattern was similar in cells incubated in Ca\textsuperscript{2+} or Sr\textsuperscript{2+}-containing media, indicating that capacitation-dependent changes related to Ca\textsuperscript{2+}-association to the plasma membrane also take place in Sr\textsuperscript{2+}-medium.

Although there is some debate regarding the positive or negative regulation Ca\textsuperscript{2+} exerts on tyrosine phosphorylation (Visconti et al., 1995; Baker et al., 2004), this event is used to verify that capacitation is taking place. A similar pattern of tyrosine phosphorylation was observed in hamster sperm incubated in medium containing any of the two divalent cations. We have previously reported this result in

**Figure 6** Sperm incubation in Sr\textsuperscript{2+}-medium and ENaC function. After a 3 h incubation under capacitating conditions in Ca\textsuperscript{2+} (Ca) or Sr\textsuperscript{2+} (Sr) medium, some wells were supplemented with 1 \textmu M amiloride (Ca...Am, Sr...Am). The level of AR was evaluated 30 min later (3.5 h) and at final incubation time (6 h). *: \( P < 0.001 \) versus Ca, Sr...Am and Sr...Ca.

**Figure 7** AR on the ZP. Pre-incubated sperm were used to inseminate salt-stored oocytes. After co-incubation and washing to remove unbound sperm, oocytes were placed in a sperm-free droplet. Two hours later, acrosomal status on motile sperm bound to the ZP was evaluated. Treatments: all the procedures were done in a medium containing Ca\textsuperscript{2+} (Ca) or Sr\textsuperscript{2+} (Sr); Sr\textsuperscript{2+}-incubated sperm were co-incubated with the oocytes in Sr\textsuperscript{2+}-medium supplemented with amiloride (Sr + Am) or Ca\textsuperscript{2+} (Sr + Ca). In some cases, amiloride (Sr...Am) or Ca\textsuperscript{2+} (Sr...Ca) was added to the sperm-free droplet, i.e. after binding *: \( P < 0.05 \) versus Ca, Sr + Am, Sr + Ca.
humans (Marín-Briggiler et al., 1999). This result is predictable since major tyrosine phosphorylation changes are associated with sperm motility, and no differences in this parameter were observed in hamster or human sperm (Marín-Briggiler et al., 1999; Zitta et al., 2004). Moreover, we found no differences in the rate of hyperactivated sperm or any of the motility parameters between cells incubated in Ca\(^{2+}\) or Sr\(^{2+}\) medium in humans (Marín-Briggiler et al., 1999) and mouse sperm (data not shown).

Taken together, these results suggest that Sr\(^{2+}\) and Ca\(^{2+}\) are able to sustain capacitation-associated events in the head (CTC pattern) and the tail (tyrosine phosphorylation). In the case of AR, we cannot rule out that some capacitation-associated events are taking place after Ca\(^{2+}\) is provided, but they should be necessarily late and rapid, since spontaneous AR is significantly increased 30 min after the addition of Ca\(^{2+}\) (Zitta et al., 2004).

Considering that there are two Ca\(^{2+}\) sources involved in the AR (acrosomal and extracellular), we wonder whether the inability of Sr\(^{2+}\) to sustain this event was specifically related to one of these sources. We accordingly considered the following possibilities: (a) the capacitation-associated changes that allow the divergent cation entry were not taking place in Sr\(^{2+}\) medium, (b) Sr\(^{2+}\) was unable to enter the cell, (c) Sr\(^{2+}\) entered the cell but could not trigger cation release from the acrosome or, (d) Sr\(^{2+}\) could not replace Ca\(^{2+}\) in the massive entry from the media after acrosomal store depletion.

To analyze the first possibility, sperm were capacitated under control conditions (Ca\(^{2+}\) medium) but shifted to Sr\(^{2+}\) medium by the time spontaneous AR should start. These cells did not undergo AR even 3 h later (Ca/Sr). Considering that these sperm had already undergone the capacitation-associated changes allowing divalent cation entry, option ‘a’ seemed unlikely. However, the possibility that sperm resuspension in Sr\(^{2+}\) medium would produce rapid changes that avoid AR to take place cannot be ruled out.

When the release of acrosomal Ca\(^{2+}\) was forced by TG treatment, AR took place in Sr\(^{2+}\) medium (Ca/Sr/TG) and reached control values. Moreover, when this treatment was repeated in sperm incubated in Sr\(^{2+}\) during the whole procedure (Sr/Sr/TG), a similar result was obtained, mediated by extracellular Sr\(^{2+}\) (Sr/Sr + TG + EGTA). These results suggest that Sr\(^{2+}\) is able to replace Ca\(^{2+}\) in its final massive entry, discarding option ‘d’.

Taking this into account, the ability of Sr\(^{2+}\) to open the acrosomal store was analyzed, inducing Sr\(^{2+}\) entry to the cell by depolarization. When sperm incubated in Sr\(^{2+}\) were resuspended in Na\(^{+}\)-free K\(^{+}\)-containing medium, AR took place and reached a level similar to that of controls. This result suggests that once Sr\(^{2+}\) is inside the sperm, the signaling cascade leading to AR can be completed, i.e. Sr\(^{2+}\) can induce the release of the acrosomal store, ruling out option ‘c’.

Taken together, these results leave as options either that Sr\(^{2+}\) was unable to sustain some capacitation-associated changes and/or that it could not spontaneously enter the cell as Ca\(^{2+}\) does.

While carrying out depolarization experiments, two particular events were observed: (1) capacitation was required to get high AR levels by cell depolarization, and (2) the absence of Na\(^{+}\) allowed sperm to undergo AR in Sr\(^{2+}\)-medium. At that point, we suspected that these results were probably connected to the particular process regulating sperm-specific exocytosis. Before capacitation, sperm Ca\(^{2+}\) channels are in an inactive state due to the depolarized membrane potential that would be regulated by ENaC (Florman, 1998; Hernandez-Gonzalez et al., 2006). When ENaC inactivates as a consequence of capacitation (Hernandez-Gonzalez et al., 2006), it causes membrane hyperpolarization, leaving Ca\(^{2+}\)-channels in a close state but available for opening (Florman, 1998; Arnoult et al., 1999). Considering this, we hypothesized that the absence of AR in Sr\(^{2+}\)-incubated sperm would be related to an active ENaC. When Sr\(^{2+}\)-incubated sperm were treated with a specific ENaC inhibitor, they could undergo spontaneous and ZP-induced AR, supporting this possibility. Since the capacitation-dependent inactivation of ENaC has been revealed recently (Hernandez-Gonzalez et al., 2006), the mechanisms associated with its regulation remains to be determined. The activity of ENaC is modulated by several factors, including ions, enzyme modification, hormones, lipids, trafficking, gene expression, etc. In sperm, there is only one study reporting that, like in other cell types, ENaC activity is regulated by the cystic fibrosis transmembrane conductance regulator (CFTR) (Hernandez-Gonzalez et al., 2007). However, this regulatory mechanism does...
not seem to be connected to the lack of AR in Sr$^{2+}$ medium. The ability to inhibit ENaC has been associated with the chloride channel activity of CFTR (Reddy et al., 1999). Considering that CaCl$_2$ was replaced by SrCl$_2$, no difference associated with chloride-transport is expected. Besides, no effect was observed when DIDS, an anion transporter inhibitor, was tested. A possible alternative is the direct participation of intracellular Ca$^{2+}$ in the regulation of sperm ENaC, as previously reported for collective duct cells (Gu, 2008).

We also analyzed the possible participation of other Na$^+$-transport systems previously related to capacitation, in the inability of Sr$^{2+}$-incubated sperm to undergo AR. However, neither the Na$^+$/HCO$_3^-$ co-transporter nor the Na$^+/K^+$-ATPase seemed to be implicated in the event under study. Na$^+/K^+$-ATPase has been involved in sperm membrane potential changes related to extracellular Ca$^{2+}$ (Espinosa and Darszon, 1995; Gonzalez-Martinez, 2003; Torres-Flores et al., 2008). Several differences explain this apparent contradiction: (1) we worked with a medium where Ca$^{2+}$ was replaced by Sr$^{2+}$, i.e. not in the absence of any divalent cation; (2) we tried to analyze the putative involvement of Na$^+$/K$^+$-ATPase on the differences between sperm incubated for 3 h in Ca$^{2+}$ or Sr$^{2+}$-containing media, not in changes associated with resting cells; (3) the event under study was not the same, since the previously mentioned reports analyzed the involvement of Na$^+$/K$^+$-ATPase in the hyperpolarization induced by Ca$^{2+}$ re-addition. We verified that the increase in AR produced when Ca$^{2+}$ was added to Sr$^{2+}$-incubated sperm was not blocked by ouabain (data not shown) sustaining the idea that the events studied by Gonzalez-Martinez and co-workers was different to the one analyzed here. However, there is a strong common observation: changes associated with the presence or absence of extracellular Ca$^{2+}$ are both rapid and reversible.

None of the regular markers used to analyze capacitation was able to detect differences between sperm incubated in Ca$^{2+}$ or Sr$^{2+}$-containing medium. However, cells incubated in these two media differ in their ability to undergo AR. The breaking point between capacitation and AR has been pursued for years but remains elusive. The last capacitation-related events and the first AR-triggering steps are probably overlapped or at least difficult to differentiate. Dissociation of the two processes was our goal by the time we decided to replace Ca$^{2+}$ by Sr$^{2+}$ in the incubation medium. In our previous reports, we assumed that Sr$^{2+}$-incubated sperm were capacitated. However, at this point, we favor an alternative explanation that Sr$^{2+}$-incubated sperm have probably undergone most changes associated with capacitation, the remaining ones being late, reversible and rapid.

Within the alkali earth metals, Sr$^{2+}$ is contiguous to Ca$^{2+}$ having the closest physicochemical properties. It is able to replace Ca$^{2+}$ in several cellular processes: permeates through voltage-dependent calcium channels, activates IP3 signals related to the opening of intracellular stores, replenishes these stores and is released in response to an agonist (Kwan and Putney, 1990; Gregoire et al., 1993; Marshall and Taylor, 1994; Morgan and Jacob, 1996; Hernandez-Cruz et al., 1997; Lemmens et al., 2001). However, AR is a peculiar exocytosis and as such has unique regulatory mechanisms, like the depolarized membrane potential that keeps Ca$^{2+}$-channels in a non-activable state. Considering the proposed participation of ENaC in the regulation of sperm membrane potential (Hernandez-Gonzalez et al., 2006), and the results obtained with amiloride in Sr$^{2+}$-incubated sperm, it is reasonable to hypothesize that these cells might not be hyperpolarized. If true, our results suggest that Ca$^{2+}$ present in the incubation media is involved in the regulation of sperm membrane potential changes associated with capacitation. Intracellular Ca$^{2+}$ levels are known to increase during capacitation, but the channels involved in its entry have not been identified. It is possible that Sr$^{2+}$ could not permeate into sperm through these channels or, if it does, is unable to replace Ca$^{2+}$ in some unidentified event. Any of these two possibilities would be in accordance with an active ENaC considering its possible regulation by intracellular Ca$^{2+}$ like in other cell types (Gu, 2008).

Present results give further support to the utility of Sr$^{2+}$-modified medium to functionally separate capacitation from AR. Its efficacy proved to be particularly useful to link sperm ability to bind to the ZP with tyrosine phosphorylation in the apical region of mouse sperm head (Asquith et al., 2004). This event has probably gone unnoticed under other conditions where Ca$^{2+}$-incubated sperm bound to ZP would have undergone AR.

Finally, the different experimental conditions used for measuring AR on the ZP both validate the results obtained in spontaneous AR, and revealed that the signaling cascade leading to sperm exocytosis cannot be interrupted and resumed later. This bioassay could be an interesting experimental model to figure out the precise steps involved between sperm binding to the ZP and completion of AR.

### Authors’ roles
K.Z. performed the first group of experiments together with S.B., who was responsible for the major part of the results. R.J.M.-H. carried out the last set of experiments and E.V.W. made motility analysis and a critical revision of the manuscript. P.V.M. was responsible for experimental design, data processing and writing of the manuscript.

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