Intracellular calcium and protein tyrosine phosphorylation during the release of bovine sperm adhering to the fallopian tube epithelium *in vitro*

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Abstract

In mammals, sperm adhesion to the epithelial cells lining the oviductal isthmus plays a key role in the maintenance of motility and in the selection of superior quality subpopulations. In the bovine species, heparin and other sulfated glycoconjugates powerfully induce the synchronous release of sperm adhering to tubal epithelium *in vitro* and may represent the signal which triggers release at ovulation *in vivo*. Sperm detachment may be due either to surface remodeling or to hyperactivation brought about by capacitation. In this paper, the dynamics of intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and protein tyrosine phosphorylation in sperm during and after heparin-induced release from *in vitro* cultured oviductal monolayers were assessed to determine whether this event is due to capacitation. Moreover, Ca^{2+} -ionophore A23187, thapsigargin, thimerosal and caffeine were used to determine whether $[\text{Ca}^{2+}]_i$ increase and/or hyperactivation can induce sperm release. Results showed that: 1. heparin-released sperm have significantly higher $[\text{Ca}^{2+}]_i$ than adhering sperm; 2. heparin induces a $[\text{Ca}^{2+}]_i$ elevation in the sperm head followed by detachment from the monolayers; 3. external Ca^{2+} is not required for heparin-induced release; 4. $[\text{Ca}^{2+}]_i$ increase and/or hyperactivation are unable to release sperm; and 5. heparin-released sperm have an increased level of tyrosine phosphorylated proteins compared with adhering sperm. In conclusion, although heparin is considered a long-lasting capacitation agent, it quickly modulates the capacitation of bovine sperm adhering to the fallopian epithelium, probably leading to surface remodeling and therefore to the release of sperm selected and stored within the oviduct through adhesion.

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Introduction

The mammalian oviduct acts as a functional sperm reservoir, providing a suitable environment that allows the maintenance of sperm fertilization competence until ovulation (Harper 1994). In several species, after mating, sperm are sequestered in the lower oviduct through adhesion to the epithelial cells lining its lumen (Wilmot & Hunter 1984, Smith & Yanagimachi 1990). This event is thought to prolong sperm life by delaying capacitation until unknown ovulation-associated signals induce the release of adhering sperm, allowing their progression toward the upper oviduct for fertilization (Smith & Yanagimachi 1991, Harper 1994). Adhesion to the oviduct has been shown to play a key role also in the selection of pre-existing sperm subpopulations characterized by intact acrosomes (cow: Gualtieri & Talevi 2000), an uncapacitated status (horse: Thomas *et al.* 1995, cow: Lefebvre & Suarez 1996, pig: Fazeli *et al.* 1999), low internal free

calcium content and reduced membrane protein tyrosine phosphorylation (pig: Petrunina *et al.* 2001), superior morphology (horse: Thomas *et al.* 1994), and normal chromatin structure (human: Ellington *et al.* 1998). Several lines of evidence indicate that the ability of sperm to bind to and be released from oviductal epithelium is modulated by capacitation. In fact, only uncapacitated sperm adhere to oviductal cells (Smith & Yanagimachi 1991, Thomas *et al.* 1995, Lefebvre & Suarez 1996), whereas the detachment of sperm in the periovulatory period *in vivo* (Smith & Yanagimachi 1991), as well as the spontaneous release during *in vitro* co-culture (Gualtieri & Talevi 2000), is thought to be triggered by a remodeling of the sperm plasma membrane and/or by hyperactivation brought about by capacitation. In cattle, heparin-like glycosaminoglycans, normally present in the oviductal fluid, have been regarded as potential *in vivo* capacitating agents (Parrish *et al.* 1988, 1989a). In a recent study, heparin, a glycosaminoglycan routinely used to capacitate sperm in

bovine *in vitro* fertilization (IVF), and other sulfated glycoconjugates were shown to induce the release of sperm adhering to the fallopian tube epithelium *in vitro* (Talevi & Gualtieri 2001). This represented the first demonstration of molecules able to cause the release of sperm adhering to the oviductal epithelium among all species examined so far. Since heparin-like glycosaminoglycans are present in the bovine oviductal fluid, and change in their concentrations and capacitating activities is maximal at estrus (Parrish *et al.* 1989a), sulfated glycosaminoglycans have been suggested to modulate progressively sperm capacitation, first inducing sperm release from the oviductal reservoir and then enhancing sperm fertilization ability (Talevi & Gualtieri 2001). Recently, the powerful sperm-releasing activity of sulfated glycosaminoglycans allowed us to demonstrate that the sperm subpopulation selected by adhesion to *in vitro* cultured fallopian tube epithelium has an enhanced zona pellucida binding and fertilization competence compared with the sperm subpopulation unable to adhere within the same ejaculate (Gualtieri & Talevi 2003, Talevi & Gualtieri 2004). Therefore, sperm adhesion and release can represent a crucial selective event during capacitation. Capacitation consists of a complex series of finely tuned events occurring during the sperm transit through the female reproductive tract, and that are essential for sperm to develop the ability to bind the zona pellucida, undergo the acrosome reaction and fertilize the oocyte. Two well-recognized events accompanying capacitation of mammalian sperm are an increase of sperm intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and the phosphorylation of protein tyrosine residues (Visconti *et al.* 2002). The aim of the present paper was to test the hypothesis that heparin-induced release of sperm adhering to the fallopian tube epithelium *in vitro* represents a very early event of capacitation. For this purpose, experiments were designed to study the following: 1. the modification of sperm $[\text{Ca}^{2+}]_i$ and protein tyrosine phosphorylation levels during and/or after heparin-induced sperm release; 2. the role played by extracellular Ca^{2+} in heparin-induced sperm release; and 3. the effect of the pharmacologic agents Ca^{2+} -ionophore A23187, thapsigargin, thimerosal and caffeine that induce $[\text{Ca}^{2+}]_i$ increase and/or hyperactivation (Ho & Suarez 2001) on the release of sperm adhering to the fallopian tube *in vitro*. The main findings demonstrate that heparin-induced release is accompanied by increase of both sperm $[\text{Ca}^{2+}]_i$ and tyrosine phosphorylation, supporting the hypothesis that heparin is able to modulate quickly the capacitation of adhering sperm.

Materials and Methods

Chemicals

Bovine serum albumin (BSA) (fraction V), heparin (sodium salt, purified from porcine intestinal mucosa, H3393), dextran sulfate, fucoidan, calcium ionophore A23187,

caffeine, thimerosal, Percoll, gelatin and Hoechst 33342 were obtained from Sigma; M199, fetal calf serum (FCS), gentamycin, Fungizone, HEPES and sodium bicarbonate, from Gibco (Milan, Italy); Fluo 3 AM, from Molecular Probe (Milan, Italy); thapsigargin, from Alomone Labs (Milan, Italy); and antiphosphotyrosine monoclonal antibody (clone 1G2) and TRITC-conjugated goat antimouse antibody, from Chemicon (Milan, Italy). Reagents and water for preparation of salines and culture media were all cell culture tested.

Oviduct monolayers

Oviducts were collected at the time of slaughter and transported to the laboratory in Dulbecco's PBS (D'PBS) supplemented with 50 µg/ml gentamycin at 4°C. Laminae of epithelial cells were recovered from oviducts of single animals by squeezing and cultured in M199 supplemented with 50 µg/ml gentamycin, 1 µg/ml Fungizone and 10% FCS, as previously described (Gualtieri & Talevi 2000). Bovine oviductal epithelial cells (BOEC) were cultured in 10 cm Petri dishes (Falcon; Becton Dickinson Milan, Italy) for 24–48 h and then transferred into four-well tissue culture dishes (Nunc, Roskilde, Denmark) with 12 mm, gelatin-coated, German glass, round cover slips on the well bottom. Fresh media changes were performed every 48 h. Cell confluence was attained in about 7–10 days. Monolayers were used within 24–48 h after attainment of cell confluence. Within each experiment, BOEC monolayers from a single individual were washed three times in modified Tyrode's albumin lactate pyruvate medium (sperm-TALP: Parrish *et al.* 1988, modified as described in Paula-Lopes *et al.* 1998), and left in this medium until sperm addition (1–3 h).

Sperm preparation

Frozen bovine semen from three bulls (0.5 ml straws; approximately 40×10^6 sperm per straw), obtained from Semen Italy (San Giuliano Saliceta, Modena, Italy), was used in all experiments. Straws were thawed in a water bath at 38°C for 30 s and the semen was laid upon a discontinuous (90/40) Percoll gradient with or without 10 µg/ml Hoechst 33342, and centrifuged for 30 min at 180 g. The supernatant was removed and the pellet, resuspended in 2 ml BSA-free sperm-TALP, was centrifuged at 180 g for 10 min, and resuspended in 200 µl BSA-free sperm-TALP. Aliquots of recovered sperm were assessed for concentration and percent motility at the hemocytometer on a microscope stage heated to 38.5°C.

Effect of extracellular Ca^{2+} on sperm release

Ca-free sperm-TALP was prepared omitting CaCl_2 and adding EGTA at 2 mM final concentration. Sperm suspension recovered after Percoll centrifugation was added to BOEC monolayers cultured on gelatin-coated, 12 mm, round cover slips in NUNC four-well plates in 750 µl sperm-TALP at a concentration of $2\text{--}3 \times 10^6$ /well, and

incubated at 39°C, 5% CO₂ in air, 95% humidity, for 60 min. After unbound sperm removal, all wells with adhering sperm were extensively washed with sperm-TALP or with Ca-free sperm-TALP. Control wells were left in these media for 10 min, whereas experimental wells were treated with heparin 100 µg/ml for 10 min. At the end of treatment, sperm-oviductal co-cultures were fixed and analyzed to quantify number of bound sperm, as previously described (Talevi & Gualtieri 2001). Briefly, monolayers grown on cover slips, inseminated with Hoechst-labeled sperm, were fixed in glutaraldehyde 2.5% in PBS, for 1 h at 20–25°C, extensively washed and mounted with the same buffer on a glass slide with cells facing up. For each well, fields of 0.286 mm² were acquired at a Zeiss Axioplan microscope equipped with phase-contrast, fluorescence and Nomarsky optics, by means of an Optronix camera and KS 300 software (Zeiss, Milan, Italy). The number of bound sperm was determined by analyzing ten fields of 0.286 mm² for each well.

[Ca²⁺]_i determinations

Sperm suspensions (2–5 × 10⁶/ml) in BSA-free sperm-TALP were added with 2.5 µM cell-permeable fluo-3FF/AM, 0.02% Pluronic F-127, and coincubated in the dark for 30 min with BOEC monolayers previously washed with BSA-free sperm-TALP at 39°C, 5% CO₂ in air, 95% humidity. At the end of coincubation, the unbound sperm fraction was removed, and after extensive washings with sperm-TALP to eliminate excess dye, the BOEC monolayers with bound spermatozoa were incubated for an additional 20 min at 39°C to allow de-esterification of the fluo-3FF/AM to its Ca²⁺-sensitive form, fluo-3FF. In experiments on sperm Ca²⁺ dynamics in response to heparin, Ca²⁺-ionophore A23187, thimerosal, caffeine or thapsigargin, cover slips with co-cultures were removed from the incubator, transferred in 0.5 ml sperm-TALP in a circular cover-slip chamber, and placed on a thermal plate at 38.5°C on the microscope stage. In experiments designed to analyze the sperm [Ca²⁺]_i, dye-loaded heparin-released sperm were allowed to attach to the glass by their heads in BSA-free sperm-TALP in the cover slip-chamber, whereas parallel dye-loaded sperm adhering to monolayers cultured on glass were directly mounted in the cover-slip chamber. Intracellular Ca²⁺ changes detected by means of fluorescence intensity of fluo3-FF were analyzed with a computer-controlled photo-multiplier system. Briefly, a digital video microscopy system was based on a Zeiss Axiovert 135 microscope. Stroboscopic illumination was provided by a 100W xenon arc flash lamp. Light passed through an excitation filter (D500/20), a dichroic mirror (515DCLP), and emission filter (D535/30) to an ORCA-100 Hamamatsu 12-bit digital camera, controlled by a Macintosh G3 workstation. The computer was used to control the microscopy system and to perform all the image acquisitions/elaborations by the Openlab software (Improvision, Coventry, UK).

In experiments performed to analyze the sperm [Ca²⁺]_i dynamics, relative fluorescence intensity (RFI) was calculated by normalizing the fluorescence intensity of the sperm head and midpiece after addition of heparin, or Ca²⁺-ionophore A23187, thimerosal, caffeine or thapsigargin (F1), against sperm basal fluorescence levels at time 0 (F0) to obtain reliable information regarding transient [Ca²⁺]_i changes from baseline values (RFI = (F1 – F0)/F0). Relative fluorescence intensity was averaged for all recorded cells. In experiments designed to analyze adhering sperm [Ca²⁺]_i changes during heparin-induced release, heparin at 100 µg/ml final concentration was added at 10–20 s after the beginning of acquisitions. Images were typically captured at intervals of 2–10 s for 2–5 min. In experiments designed to analyze the [Ca²⁺]_i in different sperm samples, the fluorescence intensity of sperm was expressed in arbitrary units calculated by subtracting the intensity of background fluorescence measured on areas free of spermatozoa in each record field. In all experiments, single-cell analysis was performed by drawing an ellipse around the head and midpiece of each sperm, and the mean intensity was obtained for all images in the time series. The image series was then analyzed, and if the defined area no longer contained the entire sperm head and midpiece, the data were excluded from analysis.

Treatment of sperm with pharmacologic agents

Sperm adhering to BOEC monolayers were treated with Ca²⁺-increasing and/or sperm hyperactivating agents, that is, Ca²⁺ ionophore A23187, thapsigargin, caffeine and thimerosal, to determine their effectiveness at inducing sperm release. Ca²⁺-ionophore A23187 was tested at 1–10 µM, thapsigargin at 5 µM, caffeine at 10 mM and thimerosal at 50 µM. Ca²⁺-ionophore A23187 and thapsigargin were dissolved in DMSO, and aliquots were stored frozen at –30°C. Their working solutions were prepared immediately before addition to the sperm suspension by diluting the stock solution in Ca²⁺-free sperm-TALP. Thimerosal was prepared in Ca²⁺-free sperm-TALP just before use. Caffeine was dissolved in Ca²⁺-free sperm-TALP by heating at 70°C (Patel *et al.* 1997).

Immunocytochemistry of sperm tyrosine phosphorylated proteins

The immunocytochemical localization of tyrosine phosphorylated proteins was performed on the following: 1. the initial sperm suspension recovered after Percoll centrifugation; 2. unbound sperm collected after 1 h co-culture with monolayers; 3. bound sperm adhering to monolayers cultured on gelatin-coated, round cover slips at 1 h co-culture; and 4) bound sperm collected at 5 min after induction of release by addition of heparin at 100 µg/ml. Sperm suspensions were spotted on glass slides and air-dried, whereas sperm adhering to monolayers were directly air-dried. Samples were fixed in methanol for 10 min; washed for 3 × 5 min in D'PBS; blocked in 50%

goat serum, Triton-X100 0.1% in D'PBS, overnight at 4 °C; washed for 2 × 5 min in Triton-X100 0.1% in D'PBS; incubated with mouse antiphosphotyrosine monoclonal antibody 10 µg/ml in 1% goat serum, Triton-X100 0.1% in D'PBS, for 1 h at 37 °C; washed for 3 × 10 min in Triton-X100 0.1% in D'PBS; incubated with TRITC-conjugated goat antimouse immunoglobulin (Ig) G 10 µg/ml in 1% goat serum, Triton-X100 0.1% in D'PBS, for 1 h at 37 °C; washed for 3 × 10 min in Triton-X100 0.1% in D'PBS; and mounted in glycerin 90% in D'PBS. Images were acquired at a Zeiss Axioplan microscope equipped with phase-contrast, fluorescence and Nomarsky optics, by means of an Optronix camera and KS 300 software (Kontron, Zeiss, Milan, Italy). For each sample, at least 200 sperm were counted in three different experiments. Specificity controls were performed by omission of the primary antibody or by saturation of the primary antibody with 20 mM *o*-phosphotyrosine.

Statistical analysis

Fluorescence intensity data of adhering and heparin-released sperm were compared by GLM procedure of ANOVA (SAS/STAT Users Guide 1988). Raw data from the immunolocalization experiment were modified by arcsine transformation to normalize data. Then, the GLM procedures were used for all analyses of variance. In the experiments for testing the influence of extracellular Ca^{2+} on sperm release, the model included the presence/absence of external Ca and heparin. For immunolocalization data, the model included incubation time (0 and 1 h), heparin treatment and sperm condition (adhering and released). Pairwise comparisons of means were made with Tukey's honestly significant difference.

Results

Sperm $[\text{Ca}^{2+}]_i$ and heparin-induced release

Experiments to analyze $[\text{Ca}^{2+}]_i$ during heparin-induced release of sperm adhering to oviductal monolayers were designed as described in the Materials and Methods section.

Cells of the oviductal monolayers did not show any detectable fluorescence under the dye-loading conditions used, whereas adhering sperm showed a slight fluorescence at the level of the sperm head. When 100 µg/ml heparin were added to dye-loaded co-cultures, all adhering sperm were released within 10 min under incubator conditions. This demonstrates that dye loading did not interfere with the sperm ability to respond to heparin. In a first series of experiments, basal fluorescence of adhering sperm (control) was registered in co-cultures in BSA-free sperm-TALP. Single-cell analysis showed a stable level of fluorescence over the time series with a progressive slight decrease of fluorescence probably due to photobleaching. After heparin treatment, released sperm were recovered and allowed to attach to a glass slide in BSA-free

sperm-TALP. The slides were then immediately registered for fluorescence. Under these conditions, 80–90% of adhering sperm detached from the monolayer. After subtraction of the background, the mean fluorescence intensity of released sperm was increased about 1.4-fold compared with the basal levels registered at the same time in control sperm (mean ± S.D.: 1018 ± 335 vs 756 ± 189 ; $P < 0.0001$; $n = 3$; sperm analyzed = 150).

For study of the $[\text{Ca}^{2+}]_i$ dynamics during sperm detachment from monolayers, heparin addition to co-cultures was performed directly in the imaging chamber during the acquisitions. The graph and corresponding micrographs in Fig. 1 show the changes of the fluorescent signal of a sperm adhering to an oviductal monolayer in a representative experiment. Heparin at 100 µg/ml was added at about 20 s after starting the acquisition. Fluorescence started to increase 10–20 s after heparin addition at the level of the sperm head and continued until detachment from the monolayer, which occurred 120–130 s later. Sperm release was complete in the areas of co-cultures unexposed to the excitation light, but it was far less efficient in the imaged areas probably due to the phototoxic effects of the excitation light. Single-cell analysis of adhering sperm that were released after heparin addition revealed peak values of 0.8 ± 0.22 RFI (mean ± S.D.; $n = 6$; sperm analyzed = 87).

Effect of external Ca^{2+} on sperm release

Preliminary experiments showed that incubation of oviductal monolayers in Ca^{2+} -free sperm-TALP for more than 15 min causes the dissociation of cells from the monolayer. Therefore, to investigate the involvement of external Ca^{2+} in heparin-induced release, sperm were first co-cultured with oviductal monolayers in sperm-TALP; after removal of unbound sperm, the following co-cultures were treated: 1) sperm-TALP; 2) Ca^{2+} -free sperm-TALP; 3) heparin 100 µg/ml in sperm-TALP; and 4) heparin 100 µg/ml in Ca^{2+} -free sperm-TALP. All co-cultures were fixed for quantitative analysis 10 min after heparin addition. As shown in Fig. 2, treatment with Ca^{2+} -free sperm-TALP did not induce sperm release; addition of heparin in Ca^{2+} -free sperm-TALP caused sperm release at an extent comparable to that recorded in sperm-TALP.

Effect of pharmacologic agents on sperm $[\text{Ca}^{2+}]_i$ and release

Experiments were designed to test the ability to evoke sperm release from oviductal monolayers by increasing either sperm $[\text{Ca}^{2+}]_i$ or flagellar beat frequency by means of pharmacologic agents. Hence, sperm oviductal co-cultures were treated with Ca^{2+} -ionophore A23187, thapsigargin, thimerosal or caffeine, as described in the Materials and Methods section. All pharmacologic agents tested were able to increase sperm $[\text{Ca}^{2+}]_i$ but failed to induce sperm release. Parallel samples not exposed to the dye were treated with pharmacologic agents in the

Figure 1 Effect of heparin (100 µg/ml) on [Ca²⁺]_i of sperm adhering to oviductal monolayers. (A) Graph shows a typical [Ca²⁺]_i increase, expressed in relative fluorescence units, of the sperm depicted in the upper left corner of panel B in a representative experiment. The decline in fluorescence at about 160 s was due to the detachment of the imaged sperm from the oviductal monolayer. (B) Time-lapse imaging of adhering sperm during heparin-induced release. Oviductal cells were not visualized under the fluo-3FF/AM charging and acquisition conditions utilized. Numbers at the lower left corner of figures represent time in seconds. Bar = 10 µm.

cover-slip chamber and observed at intervals of 10 min for 1 h under phase-contrast microscopy to ascertain: 1. that dye loading did not interfere with the ability of sperm to be released by the agent; and 2. that an eventual releasing activity of the pharmacologic agents needed a longer incubation time to occur. Even under these conditions, all agents failed to induce the release of adhering sperm. Figure 3 shows the increase of fluorescence caused by 10 µM ionophore in five adhering sperm during a 40 s acquisition (one frame/5 s) in a representative experiment. Ca²⁺-ionophore caused a sharp fluorescence increase (peak, mean ± s.d.: 1.5 ± 0.41 RFI; *n* = 5; sperm analyzed = 65) in both the sperm head and midpiece that started about 5 s after addition of the

reagent. However, this treatment caused immobilization of all adhering sperm. Repeated resuspensions of the medium in the cover-slip chamber after ionophore treatment did not displace sperm, showing that they were still firmly bound to monolayers. In the next series of experiments, treatment with 1 µM ionophore maintained sperm motility, causing sperm [Ca²⁺]_i elevations without inducing sperm release from the monolayer (data not shown).

Thapsigargin, a Ca²⁺-ATPase inhibitor that increases [Ca²⁺]_i through release from internal stores (Thastrup 1990), has been reported to increase sperm [Ca²⁺]_i in several species and to induce hyperactivation in bovine sperm (Ho & Suarez 2001). Addition of 5 µM thapsigargin to sperm bound to oviductal monolayers caused a sudden increase of sperm [Ca²⁺]_i (peak, mean ± s.d.: 2.1 ± 0.8 RFI), which recovered to basal values in about 120 s (Fig. 4A) (*n* = 4; sperm analyzed = 72).

Thimerosal, a drug able to release [Ca²⁺]_i from both inositol trisphosphate (IP₃)- and ryanodine-operated stores, has been reported to induce elevations of [Ca²⁺]_i and hyperactivation in bovine sperm (Ho & Suarez 2001). Treatment of sperm adhering to monolayers with thimerosal 50 µM caused a rapid [Ca²⁺]_i increase that reached maximum value at about 60 s (peak, mean ± s.d.: 0.8 ± 0.4 RFI) (Fig. 4B) (*n* = 4; sperm analyzed = 67).

Treatment of co-cultures with caffeine, an agent that releases [Ca²⁺]_i from ryanodine-operated stores and hyperactivates bovine sperm (Ho & Suarez 2001), induced [Ca²⁺]_i elevations (peak, mean ± s.d.: 1.6 ± 1.0 RFI)

Figure 2 Effect of Ca-free medium on the release of sperm adhering to oviductal monolayers (*n* = 3; means ± s.d.). In all samples, sperm-oviduct adhesion was performed for 1 h in sperm-TALP, and then unbound sperm were removed. Control: 10 min in sperm-TALP; Ca-free: 10 min in Ca-free sperm-TALP; heparin: 10 min in sperm-TALP plus 100 µg/ml heparin; heparin in Ca-free: 10 min in Ca-free sperm-TALP plus 100 µg/ml heparin (control vs heparin; Ca-free vs heparin in Ca-free: *P* < 0.0001).

Figure 3 Effect of Ca^{2+} ionophore A23187 ($10\ \mu\text{M}$) on $[Ca^{2+}]_i$ of sperm adhering to oviductal monolayers. (A) Graph shows the $[Ca^{2+}]_i$ increase expressed in relative fluorescence units of five sperm imaged in panel B in one representative experiment ($n = 5$; number of sperm analyzed was 65). Shaded and unshaded symbols indicate each sperm and relative $[Ca^{2+}]_i$ trace. (B) Time-lapse imaging of adhering sperm during ionophore treatment. Sperm remained firmly bound to the oviductal monolayers after treatment. Oviductal cells were not visualized under the fluo-3FF/AM charging and acquisition conditions utilized. Numbers at the upper left corner of figures represent time in seconds. Bar = $10\ \mu\text{m}$.

(Fig. 4C) ($n = 4$; sperm analyzed = 93). Although caffeine was the hyperactivating agent that more effectively promoted the augmentation of adhering sperm flagellar beat frequencies, it failed to induce the release of adhering sperm under our experimental conditions.

Pairwise comparisons of mean peak values recorded after application of pharmacologic agents demonstrated that thimerosal was the least effective $[Ca^{2+}]_i$ increasing agent ($P < 0.01$).

Immunocytochemistry of sperm tyrosine phosphorylated proteins

Bovine sperm processed for the immunocytochemical localization of tyrosine phosphorylated proteins exhibited the following three different labeling patterns (Fig. 5): 1. equatorial segment (pattern E); 2. acrosome (pattern A); and 3. equatorial segment and acrosome (pattern EA). Specificity controls performed either by omission of the primary antibody or by saturation of the primary antibody with 20 mM α -phosphotyrosine were both negative. For study of the dynamics of tyrosine phosphorylation during capacitation, the following samples were analyzed: 1. initial sperm suspension recovered after Percoll centrifugation at times 0 and 1 h; 2. sperm adhering to monolayers at 1 h of co-culture; and 3. initial suspension and released sperm at 1 h of culture in sperm-TALP plus 5 min treatment with heparin $100\ \mu\text{g/ml}$. Preliminary findings showed that labeling of the equatorial segment was the first pattern observed during capacitation, whereas labeling of the acrosome alone or of both the equatorial segment and acrosome was observed only at longer capacitation times.

Therefore, since A and EA may represent more advanced stages of capacitation than E, these patterns were grouped together as shown in Table 1. Data on control sperm showed a general progression both in the number of labeled sperm and in the labeling pattern. In fact, at 0 h, 4% of sperm were labeled and showed pattern E, whereas, at 1 h, labeled sperm increased at 41%, and both patterns E and A plus EA were observed (Table 1). Moreover, treatment of this sample for 5 min with heparin increased the percentage of labeled sperm to 75%. By contrast, only 2% of adhering sperm were labeled at 1 h of co-culture and showed exclusively the E pattern. Treatment for 5 min with heparin caused the release of adhering sperm and the increase of labeled sperm to 15% (Table 1).

Discussion

The epithelial cells of the isthmic portion of mammalian fallopian tube can select a fraction of uncapacitated sperm and maintain their fertile life by delaying capacitation until unknown ovulation-associated signals, inducing sperm hyperactivation and/or surface remodeling, ultimately release selected and stored sperm for fertilization (DeMott & Suarez 1992, Smith 1998). Since sperm adhesion to oviductal epithelial cells is one of the first events occurring during capacitation, it is likely that some mechanisms of capacitation are modulated through such cell-cell interaction.

In the bovine species, an acrosome-intact sperm subpopulation endowed with a superior zona pellucida binding and fertilization competence can be selected by

Figure 4 Mean [Ca²⁺]_i increases of sperm adhering to oviductal monolayers during treatment with the pharmacologic agents (A) thapsigargin (5 μM), (B) thimerosal (50 μM), and (C) caffeine (10 mM).

adhesion to *in vitro* cultured oviductal monolayers, and readily and synchronously released by nanomolar to micromolar amounts of the sulfated glycoconjugates heparin, fucoidan and dextran sulfate (Gualtieri & Talevi 2000, 2003, Talevi & Gualtieri 2001, 2004). On this basis, heparin-like glycosaminoglycans have been suggested to represent the physiologic signal for the release of sperm stored in the oviductal reservoir in the bovine species.

The aim of the present paper was to determine whether heparin-induced release of selected sperm represents a very early event of sperm capacitation within the female reproductive tract. To this end, experiments were designed to study sperm [Ca²⁺]_i and protein tyrosine phosphorylation, two well-recognized markers of sperm capacitation, during heparin-induced release of bovine sperm adhering to the fallopian tube epithelium *in vitro*.

Figure 5 Labeling patterns of bovine sperm processed for the immunolocalization of tyrosine phosphorylated proteins. (A) equatorial segment (pattern E), (B) acrosome (pattern A), (C) equatorial segment and acrosome (pattern EA). Bar = 4 μm.

Main results indicate the following: 1. [Ca²⁺]_i increase accompanies heparin-induced sperm release; 2. heparin-induced sperm release does not require extracellular Ca²⁺; 3. triggering of sperm hyperactivation and/or [Ca²⁺]_i elevations by means of pharmacologic agents fails to elicit release of sperm adhering to the fallopian tube *in vitro*; and 4. adhesion to the oviduct *in vitro* maintains a low level of protein tyrosine phosphorylation in sperm, whereas heparin-released sperm have increased protein tyrosine phosphorylation.

Heparin, a well-known capacitating molecule routinely used in bovine IVF (Parrish *et al.* 1988), is similar to endogenous glycosaminoglycans of the oviductal fluid, whose concentration peaks around the time of ovulation (Parrish *et al.* 1989a). Several studies have shown that incubation with heparin for at least 4 h is required to induce capacitation and to increase sperm [Ca²⁺]_i, cAMP,

Table 1 Labeling patterns of bovine sperm processed for the immunolocalization of tyrosine phosphorylated proteins (n = 3; means $\pm$ s.d.). Control 0 and 1 h: sperm suspension at 0 and 1 h of incubation in sperm-TALP. 1 h + Heparin: sperm suspension at 1 h of incubation in sperm-TALP followed by treatment with heparin 100 μ g/ml for 5 min. Bound 1 h: sperm adhering to oviductal monolayers at 1 h of co-culture in sperm-TALP. Heparin-released: sperm co-cultured for 1 h in sperm-TALP and released by treatment with heparin 100 μ g/ml for 5 min.

	Control 0 h	Control 1 h	1 h + Heparin	Bound 1 h	Heparin-released
Labeled sperm (%)	4 $\pm$ 3 ^a	41 $\pm$ 8 ^{bce}	76 $\pm$ 16 ^d	2 $\pm$ 2 ^{fg}	15 $\pm$ 4 ^h
Pattern E (%)	4 $\pm$ 3 ^a	14 $\pm$ 3 ^{bc}	24 $\pm$ 7	2 $\pm$ 2 ^{de}	15 $\pm$ 4 ^f
Pattern A + EA (%)	0 ^a	27 $\pm$ 5 ^{bce}	51 $\pm$ 9 ^d	0 ^f	0

a,b,c,d,e,f and g,h Values within rows differ significantly ($P < 0.01$).

Labeling patterns: E, equatorial segment; A, acrosome; EA, equatorial segment and acrosome.

intracellular pH and protein tyrosine phosphorylation (Parrish *et al.* 1988, 1989b, 1994, Galantino-Homer *et al.* 1997, Visconti *et al.* 1998). On the other hand, heparin has an immediate effect on release of sperm adhering to the oviductal epithelium *in vitro* (Talevi & Gualtieri 2001). If release is due to the capacitating activity of heparin, this means that some events of capacitation may occur very rapidly. Present results on sperm $[Ca^{2+}]_i$ determinations agree with this hypothesis. In fact, heparin increases the $[Ca^{2+}]_i$ in the head of adhering sperm until they detach from the monolayer; moreover, heparin-released sperm have significantly higher $[Ca^{2+}]_i$ than adhering sperm. Previous studies in horses demonstrated that adhesion to oviductal cells or to apical plasma membrane preparations is responsible for the maintenance of low sperm $[Ca^{2+}]_i$ and that spontaneously released sperm have $[Ca^{2+}]_i$ about three times higher than adhering sperm (Dobranski *et al.* 1996, 1997). The maintenance of low $[Ca^{2+}]_i$ in adhering sperm may account for the reported ability of oviduct adhesion to prolong sperm fertile life and delay capacitation. On the other hand, the observation that released sperm have higher $[Ca^{2+}]_i$ than bound sperm might be explained by the removal of the inhibitory influence of oviduct adhesion on sperm $[Ca^{2+}]_i$. Results of the present study, in which the $[Ca^{2+}]_i$ dynamics of single adhering sperm was continuously monitored during heparin-induced release, demonstrate for the first time that $[Ca^{2+}]_i$ increase precedes the release of adhering sperm. Quantitative experiments on heparin-induced sperm release in Ca^{2+} -free sperm-TALP were designed to determine whether sperm $[Ca^{2+}]_i$ increase is caused by the entry of extracellular Ca^{2+} from the external medium or by release from intracellular stores. Data indicate that such an early capacitation event does not require extracellular Ca^{2+} but may be due to release from internal stores. This finding agrees with recent evidence of the existence of sperm internal Ca^{2+} stores and their involvement in the regulation of sperm functions (Walensky & Snyder 1995, Ho & Suarez 2001, Rossato *et al.* 2001). Interestingly, in the bovine species, an IP3-gated Ca^{2+} store has been shown to be involved in the initiation of hyperactivated motility (Ho & Suarez 2001). Moreover, heparin has been reported to cause a sudden increase of adhering sperm flagellar beat followed by detachment of sperm from the oviductal monolayers (Talevi & Gualtieri 2001), and to induce

hyperactivation of free-swimming sperm (Chamberland *et al.* 2001). Therefore, it can be hypothesized that addition of heparin to sperm adhering to oviductal monolayers induces first the release of Ca^{2+} from internal stores accompanied by the augmentation of flagellar beat frequencies and then sperm detachment.

Experiments with pharmacologic agents to induce sperm $[Ca^{2+}]_i$ elevations artificially and/or hyperactivation demonstrated that neither $[Ca^{2+}]_i$ elevation nor hyperactivation can cause sperm release. Capacitation is supposed to induce the release of sperm adhering to the fallopian tube epithelium, either by a rapid remodeling of the sperm plasma membrane, which involves the inactivation of adhesion molecules directed toward the oviductal epithelium, or merely by inducing sperm hyperactivation, which provides an additional force sufficient to release sperm from their cellular contacts (Smith & Yanagimachi 1991, DeMott & Suarez 1992, Lefebvre & Suarez 1996). In the present paper, several agents were selected for their reported ability to increase sperm $[Ca^{2+}]_i$ (Ca^{2+} ionophore A23187) or both $[Ca^{2+}]_i$ and hyperactivation in free-swimming sperm (thapsigargin, thimerosal and caffeine: Ho & Suarez 2001). However, under our experimental conditions, pharmacologic agents failed to release sperm, although they were able to increase sperm $[Ca^{2+}]_i$, and, at least as regards caffeine, to induce a clear increase of sperm flagellar beat frequency. Overall, present findings support the hypothesis that heparin-induced sperm release is due to a rapid remodeling of the plasma membrane that leads to a loss of affinity for the molecules involved in such cell–cell interaction.

Several studies have shown the involvement of the cAMP/protein kinase A pathway in the tyrosine phosphorylation of specific subsets of sperm proteins, as occurs during capacitation (Visconti *et al.* 1995, Galantino-Homer *et al.* 1997, Gadella & Harrison 2000). In particular, sperm protein tyrosine phosphorylation is involved in different steps of capacitation, such as the development of hyperactivated motility and the induction of acrosome reaction (Flesch & Gadella 2000). Present findings indicate that thawed frozen bull sperm are mostly uncapacitated at time 0, whereas, at longer times, adhesion to the oviductal epithelium maintains low levels of tyrosine phosphorylation compared with those detected in free-swimming sperm. This agrees with recent reports in other

species (Petrunkina *et al.* 2001, 2003) demonstrating that oviduct adhesion selects sperm with suppressed tyrosine phosphorylation and stores them in such an uncapacitated condition, thus prolonging sperm fertility. Moreover, in the bovine species, heparin treatment of both free-swimming and adhering sperm quickly triggers tyrosine phosphorylation. Interestingly, the rate of tyrosine phosphorylation in response to heparin in adhering sperm is faster than in free-swimming sperm, suggesting that the suppression of capacitation caused by adhesion to the oviduct may render the adhering sperm subpopulation more responsive to capacitation signals. On the other hand, it is also possible that oviduct adhesion selects a pre-existing sperm subpopulation endowed with higher responsiveness to heparin. Whatever the case, this finding may be related to the superior sperm–zona pellucida binding and fertilization competence of oviduct-selected sperm (Gualtieri & Talevi 2003).

In conclusion, the present data on sperm [Ca²⁺]_i and tyrosine phosphorylation dynamics during heparin-induced release from *in vitro* cultured tubal epithelium add new information suggesting that adhesion to the oviduct suppresses capacitation, and that release of adhering sperm is due to a very early event of capacitation that remodels the sperm surface, leading to a rapid loss of affinity for the oviductal epithelium.

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