

Signal transduction mechanisms involved in *in vitro* ram sperm capacitation

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Abstract

We validate the chlortetracycline (CTC) technique for the evaluation of capacitation and acrosome reaction-like changes in ram sperm, carrying out a double estimation of the acrosome status after treatment with lysophosphatidylcholine, using fluorescein isocyanate (FITC)-RCA/ethidium homodimer 1 (EthD-1) and CTC/EthD-1. Highly consistent results and a positive correlation between the results of acrosome-reacted sperm evaluated with both techniques were obtained. In this study, we evaluate the effects of ram sperm capacitation of BSA, Ca^{2+} , NaHCO_3 and cAMP agonists and their influence on the associated protein tyrosine phosphorylation. We found a time-dependent increase in capacitation related to protein tyrosine phosphorylation, either in the absence or the presence of BSA. The addition of an increasing concentration of cholesterol to samples containing BSA did not influence results. The effect of bicarbonate was concentration-dependent, with a significantly lowered value of non-capacitated sperm in the presence 18 and 25 mM. The addition of extracellular calcium did not significantly increase either the proportion of capacitated sperm or the protein tyrosine phosphorylation signalling, although a significantly higher value of acrosome-reacted sperm was found in samples containing 4 mM Ca^{2+} . cAMP agonists increased capacitated sperm and protein tyrosine phosphorylation signalling. The inhibition of protein kinase A by H-89 caused a decrease in sperm capacitation. Addition of a calcium-entry blocker (Verapamil; Sigma) did not influence results, which suggests that the calcium entry blocker was unable to inhibit the calcium influx associated with capacitation in ram sperm. Our findings might benefit our understanding of the biochemical mechanisms involved in mammalian sperm capacitation and ultimately, fertility.

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Introduction

In mammals, freshly ejaculated spermatozoa cannot fertilize mature oocytes; rather they become fertilization competent in the female genital tract through the time-dependent process of capacitation (Yanagimachi 1994). First described by Austin (1951, 1952) and Chang (1951, 1955), capacitation is a complex set of modifications that sperm undergo in the female reproductive tract whereby sperm acquire the ability to fertilize an oocyte. The duration of capacitation varies among species and ranges from 1–2 h in sheep and rat to 10–12 h in ferret (Austin 1970). The physiological, biochemical and biophysical changes that occur during capacitation include alterations in metabolism and intracellular ion concentrations (Yanagimachi 1994, Visconti *et al.* 1995a), membrane fluidity (Wolf *et al.* 1986, Harrison & Miller 2000), membrane hyperpolarization (Zeng *et al.* 1995), intracellular pH (Zeng *et al.* 1996), cAMP

concentration (White & Aitken 1989, Fraser & Monks 1990, Visconti *et al.* 1997, 1998), protein tyrosine phosphorylation (Visconti *et al.* 1995b) and the concentrations of reactive oxygen species (de Lamirande and Gagnon 1993, Aitken *et al.* 1995, Herrero *et al.* 1999).

Capacitation occurs *in vitro* in defined media (Yanagimachi 1994) whose compositions are based on the electrolyte concentration of oviductal fluid. Typically, capacitation media contain energy substrates (e.g. pyruvate, lactate and glucose), a cholesterol acceptor (usually serum albumin), NaHCO_3 , Ca^{2+} , low K^+ and physiological Na^+ , some of which play an important role in the capacitation process.

In several mammalian species phosphorylation of tyrosine residues on a subset of proteins is positively correlated with the capacitated state of sperm (Visconti *et al.* 1995a, 1999b, Galantino-Homer *et al.* 1997, Osheroff *et al.* 1999, Tardif *et al.* 2001, Pommer *et al.* 2003). The increase in protein tyrosine phosphorylation

associated with capacitation is highly species-dependent because regulators of capacitation, such as calcium, BSA and sodium bicarbonate have effects that differ among species (Visconti *et al.* 1995a, Carrera *et al.* 1996, Kulanand & Shivaji 2001, Tardif *et al.* 2003, Baker *et al.* 2004). For example, in mouse, sperm capacitation is dependent on the presence of BSA, bicarbonate and calcium (Visconti *et al.* 1995a); yet, BSA is not necessary for the capacitation of boar sperm *in vitro* (Tardif *et al.* 2003). Similarly, in boar (Tardif *et al.* 2003) and hamster (Visconti *et al.* 1999b, Kulanand & Shivaji 2001), capacitation-associated protein tyrosine phosphorylation is not dependent on the presence of bicarbonate, and is negatively modulated by extracellular calcium during human sperm capacitation (Luconi *et al.* 1996, Baker *et al.* 2004).

Several studies (Aitken *et al.* 1998, Visconti *et al.* 1998, Ho & Suarez 2001) have proposed models for signalling cascades regulating capacitation in which cyclic adenosine 3'-5'-monophosphate (cAMP) is a key molecule. The downstream target of cAMP is the protein kinase A (PKA), whose activity increases during sperm capacitation (Visconti *et al.* 1997). Specifically, cAMP-activated PKA regulates capacitation-related tyrosine phosphorylation in specific proteins by modulating tyrosine kinase, protein phosphatase or both (Visconti *et al.* 1998). Visconti *et al.* (1995a) postulated that the production of cAMP is an event downstream from the site of BSA and NaHCO₃ action.

Previously, we demonstrated that protein tyrosine phosphorylation of membrane proteins is related to the capacitation state of ram spermatozoa (Perez-Pe *et al.* 2002). To our knowledge, there are no published studies of the molecular regulation mechanisms of this process in ram. Therefore in this study, we evaluated the effects on ram sperm capacitation of several components, including BSA, Ca²⁺, NaHCO₃ and cAMP agonists known to be key modulators in sperm capacitation in other species. In addition, sperm viability was simultaneously assessed in all conditions, unlike most studies on capacitation.

Materials and Methods

Sperm preparation and media

We used an artificial vagina to collect semen from eight mature (2–4 years old) *Rasa aragonesa* rams that belonged to the National Association of Rasa Aragonesa Breeding (ANGRA). Rams were kept at the Veterinary School under uniform nutritional conditions. Prior to sampling, sires underwent an abstinence period of 2 days (Ollero *et al.* 1996). To avoid individual differences, the second ejaculates from four rams were pooled and used in each assay.

In vitro capacitation

A seminal plasma-free sperm population obtained by a dextran/swim-up procedure (García-López *et al.* 1996) was used as the control sample. This procedure was performed with a swim-up medium (SM) devoid of CaCl₂ and NaHCO₃ (Grasa *et al.* 2004) that consisted of 200 mM sucrose, 50 mM NaCl, 18.6 mM sodium lactate, 21 mM HEPES, 10 mM KCl, 2.8 mM glucose, 0.4 mM MgSO₄, 0.3 mM sodium pyruvate, 0.3 mM K₂HPO₄, 1.5 UI/ml penicillin and 1.5 µg/ml streptomycin, pH 7.2 (adjusted using NaOH). The osmotic pressure was 320 mOsm/kg. For the induction of *in vitro* capacitation, aliquots of 2 × 10⁸ cells/ml were incubated at 39 °C in a humidified incubator with 5% CO₂ in air (capacitated sample). Incubations were performed using the described SM or the complete SM, which contained 3 mM CaCl₂ and 15 mM NaHCO₃, either with or without 5 mg/ml BSA. The bicarbonate concentration (15 mM) was chosen on the basis of results reported by Spilman *et al.* (1970) who found a medium value of 13 mM in the sheep oviduct fluid. Likewise, the effect of bicarbonate was evaluated up to 25 mM, as commonly used in synthetic oviduct fluid (SOF) medium (Tervit *et al.* 1972). In order to maintain the same osmolarity in all samples, NaCl was added. In the experiments carried out to evaluate the bicarbonate effect, samples were kept in closed tubes during the whole process.

To confirm the effect of BSA, we carried out some experiments containing different concentrations (5–30 µM) of cholesterol-3-sulphate sodium salt (chol – SO₄⁻, Sigma) in SM with BSA (5 mg/ml).

To evaluate the implication of the cAMP–PKA pathway, we tested the effects of dibutyryl-cAMP (db-cAMP, Sigma; 1 and 4 mM) and isobutyl-methylxanthine (IBMX, Sigma; 0.1 and 1 mM) in the presence of BSA, either with or without 3 mM Ca²⁺ and 15 mM NaHCO₃. We determined the effects of 0.05, 0.5 and 1 mM Verapamil (a calcium-entry blocker, Sigma) and 10, 50, 100 and 200 µM H-89 (a PKA inhibitor, Sigma) in a + Ca + HCO₃⁻ medium containing BSA (5 mg/ml). Solutions of db-cAMP and Verapamil were made in water. H-89 and IBMX were diluted in DMSO as a stock solution and added to the sperm sample to obtain the required concentrations, with DMSO < 1% final solution.

Acrosome reaction induction

The acrosome reaction was induced by the addition of lysophosphatidylcholine (LPC, Sigma; Parrish *et al.* 1988) to the swim-up obtained sample (control sample) or after 4-h incubation in capacitating conditions (capacitated samples). For this purpose, 5 µl LPC were added to 95 µl sperm sample (10⁸ cells/ml in a complete SM) to a final concentration of 300 µg/ml (Gomez *et al.* 1997) and samples were incubated at 39 °C (5% CO₂ in air, 100% humidity) for 20 min.

Assessment of semen parameters

Sperm concentrations were calculated in duplicate using a Neubauer's chamber (Marienfeld, Germany). Cell viability (membrane integrity) was assessed by fluorescent staining with carboxyfluorescein diacetate and propidium iodide (Harrison & Vickers 1990). Using a Nikon fluorescence microscope, we counted the number of propidium iodide-negative (membrane-intact) and -positive (membrane-damaged) spermatozoa per 100 cells. For each sample, at least 200 cells were counted in duplicate. Results are expressed as the proportion (%) of membrane-intact spermatozoa \pm S.E.M.

We used a modified version of the chlortetracycline (CTC) assay described by Ward & Storey (1984). In addition to simultaneous evaluation of cell viability and capacitation state, CTC staining was coupled with ethidium homodimer 1 staining (EthD-1, Molecular Probes Inc., Eugene, OR, USA) (Cross & Meizel 1989, Fraser *et al.* 1995). A CTC solution (750 μ M, Sigma) was prepared daily in a buffer containing 20 mM Tris, 130 mM NaCl and 5 μ M cysteine, pH 7.8, and passed through a 0.22 μ m filter (Millipore Ibérica, Madrid, Spain). EthD-1, 2 μ l (23.3 μ M) were added to 18 μ l sperm sample, and incubated at 37 °C in the dark for 10 min. Thereafter, 20 μ l CTC solution and 5 μ l of 12.2% (w/v) paraformaldehyde in 0.5 M Tris-HCl, pH 7.8, were added. At room temperature, a 4 μ l aliquot of stained sample was placed on a glass slide and mixed with 2 μ l 0.22 M triethylenediamine (DABCO, Sigma) in 9 glycerol:1 PBS. The samples were covered with 24 \times 48 mm coverslips, sealed with colourless enamel, and stored in the dark at 4 °C. For the evaluation of CTC patterns, within 12 h, we examined the samples using a Nikon Eclipse E-400 microscope under epifluorescence illumination using a V-2A filter. In addition, we assessed the EthD-1-positive cells (dead cells) with a G-2A filter in the same field, and thus, the percentage of each CTC pattern in the live sperm population was determined. All samples were processed in duplicate and we scored at least 150 spermatozoa/slide. No fluorescence was observed when CTC was omitted from the preparation. Three sperm types were estimated (Gillan *et al.* 1997): not capacitated (NC, even distribution of fluorescence on the head, with or without a bright equatorial band), capacitated (C, with fluorescence in the anterior portion of the head) and acrosome-reacted cells (AR, showing no fluorescence on the head). For treatment of results, only the live cells were taken into consideration.

The acrosome integrity was evaluated using the FITC-conjugated RCA (*Ricinus communis* lectin, Vector Laboratories, Burlingame, CA, USA) as previously described (Marti *et al.* 2000), combined with EthD-1 staining (23.3 μ M) for simultaneous evaluation of cell viability. Five microlitres of the stained sample were placed on a slide and covered with a coverslip. We determined the total number of cells by bright field in a

Nikon Labophot-2 microscope, assessing in each field the FITC-RCA and EthD-1 fluorescence under epifluorescence illumination with V-2A and G-2A filters respectively. Acrosome-intact cells lacked such fluorescence but the acrosome-reacted cells presented bright fluorescence from the tip of the head to the equatorial region. Dead cells were EthD-1 positive (red fluorescence). At least 200 cells in duplicate were counted for each sample.

Extraction of ram sperm proteins

Aliquots of 0.5 ml control or capacitated samples diluted 1:10 (10^7 cells) were centrifuged at 7500 *g* in a microfuge for 5 min at room temperature and the supernatant discarded. The resulting sperm pellet was resuspended in 100 μ l extraction medium (2% SDS, 28% sucrose, 12.4 mM TEMED (N,N,N',N'-tetramethyl-ethylenediamine), 185 mM Tris-HCl, pH 6.8 (Roldan & Harrison 1988) and immediately incubated for 5 min at 100 °C. After centrifugation at 7500 *g* for 5 min, the supernatant was recovered and 2-mercaptoethanol and glycerol were added to a final concentration of 5 and 1% respectively. We measured extracted protein concentrations, prior to the addition of 2-mercaptoethanol, using the Micro BCA Protein Assay Reagent kit (Pierce, Rockford, IL).

SDS-PAGE and immunoblotting

To visualize proteins with phosphorylated tyrosine residues, a volume of extract corresponding to equal numbers of sperm cells in each sample (1×10^7 cells) was incubated for 5 min at 100 °C before being loaded onto 4–22.5% (w/v) SDS-PAGE gels, following the Laemmli method (Laemmli 1970). To rule out the influence of differences in the amount of proteins extracted, we previously determined the protein concentration and the same protein amount of each sample was loaded and analyzed by SDS-PAGE. Separated proteins were blotted onto Immobilon-P (Millipore, Bedford, MA, USA), as previously described (Perez-Pe *et al.* 2002). Non-specific sites on the membranes were blocked for 1 h with 5% BSA in blocking buffer (Tris-HCl 10 mM pH 8, NaCl 120 mM and 0.05% Tween). The blots were incubated with MAB antiphospho-tyrosine mouse IgG1 (clone PT 66, Sigma) diluted 1:2000 for 3 h at 25 °C. After an exhaustive washing, the membranes were incubated for 1 h with a secondary goat anti-mouse alkaline-phosphatase-conjugated IgG (Sigma) diluted 1:4000 in blocking buffer. After extensive washing, the proteins that bound the antibody were visualized by incubation with a substrate mixture of nitro blue tetrazolium (0.4 mM) and 5-bromo-4-chloro-3-indolyl phosphate (0.38 mM) in 0.2 M Tris, 5 mM MgCl₂ and 0.1 M NaCl, pH 9.6. Finally, the membrane was washed in distilled water, dried and scanned. The Gel Doc

System with Molecular Analyst software (Bio-Rad) was used to quantify changes in tyrosine-phosphorylated proteins. The phosphorylation signal of some molecular regions was evaluated as volume (area \times intensity), and changes relative to control (sample without effector incubated 4 h) are presented as a percentage.

Statistical analysis

Results are presented as mean \pm S.E.M. of the number of samples indicated in each case. To determine whether there were significant differences among samples and between conditions of incubation, means were compared using ANOVA. Post hoc comparisons were made using Tukey's test. Correlations between data obtained with CTC and RCA staining techniques, and sperm treatments (incubation in capacitating conditions and LPC-induced AR) were determined using Pearson's coefficient. Software used was GraphPad InStat (San Diego, CA, USA).

Results

CTC staining is a valid technique for assessing ram sperm capacitation state

The CTC fluorescence assay was first described for the evaluation of mouse sperm capacitation state (Saling & Storey 1979, Ward & Storey 1984), and later applied to other mammals (Lee *et al.* 1987, Cormier *et al.* 1997). Likewise, several studies have shown that the CTC assay may be used to assess capacitation-like changes in ram spermatozoa (Perez *et al.* 1996, Gillan *et al.* 1997, Perez-Pe *et al.* 2002). Nevertheless, in this study, we have undertaken an initial validation of the technique for the evaluation of capacitation and acrosome reaction-like changes in ram sperm. For this purpose, we induced the acrosome reaction by adding LPC to capacitated sperm samples (Gomez *et al.* 1997) carrying out a double estimation of the acrosome status using FITC-RCA/EthD-1 and CTC/EthD-1 fluorescent staining. The FITC-RCA-labelling procedure was shown to accurately assess the acrosomal status of ram spermatozoa (Marti *et al.* 2000). The inclusion of EthD-1 simultaneously to RCA and CTC, allows specific determination in viable cells, considered here as both intact plasma and acrosomal membranes. Therefore, our data of CTC patterns indicate values in live sperm, exclusively.

As shown in Fig. 1A, assessment by CTC/EthD1 indicated that the proportion of capacitated sperm in samples incubated 4 h in capacitating conditions (the difference between 0 and 4 h incubation without LPC) was not significantly different ($P > 0.05$) to the percentage of acrosome-reacted sperm induced in this sample by addition of LPC (the difference between the acrosome-reacted sperm with and without LPC, after 4 h incubation). Furthermore, the augmentation in the

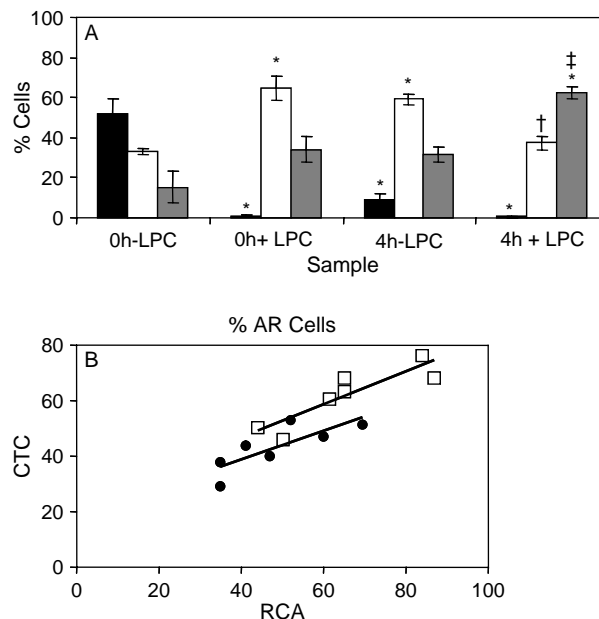


Figure 1 Validation of CTC staining for assessing ram sperm capacitation state. (A) Effect of incubation in capacitating conditions and addition of LPC on ram sperm capacitation status evaluated by CTC/EthD-1 staining. Sperm samples were obtained by swim-up (control at 0 h) and incubated 4 h at 39 °C under 100% humidity and 5% CO₂ in complete SM (with 3 mM Ca²⁺ and 15 mM HCO₃⁻) in the presence of BSA (control at 4 h). Acrosome reaction was induced by addition of 300 µg/ml LPC and incubation for 20 min at 39 °C. Mean values \pm S.E.M. ($n = 5$). Percentage of ■ not capacitated, □ capacitated, ■ acrosome-reacted sperm. Significant differences relative to control (0 h, * $P < 0.001$) or to the LPC-free control samples (4 h, † $P < 0.01$; ‡ $P < 0.001$). (B) Correlation of acrosome-reacted sperm after incubation and addition of LPC as determined by CTC (ordinate) and FITC-RCA (abscissa) labelling. ● % acrosome reacted sperm in samples incubated 4 h in capacitating conditions; □ % acrosome reacted sperm in capacitated samples after LPC-acrosome reaction induction ($n = 7$).

capacitated staining pattern after 4 h incubation in capacitated conditions was significantly correlated ($r = 0.899$, $P < 0.05$) with the induced acrosome-reacted by LPC, both determined by CTC/EthD-1 labelling. The regression line determined by the method of least squares was $y = 0.926x + 5.49$ (data not shown). In addition, a positive correlation was found between the percentage of acrosome-reacted cells in samples incubated 4 h in capacitating conditions detected by both (RCA and CTC) stainings ($r = 0.789$, $P < 0.05$), or in the same samples after inducing the acrosome reaction by addition of LPC ($r = 0.883$, $P < 0.01$; Fig. 1B).

Effect of BSA on *in vitro* ram sperm capacitation and protein tyrosine phosphorylation

In a previous study, we showed that ram sperm capacitation induced *in vitro* in a medium containing calcium and bicarbonate without BSA promoted an increase in protein tyrosine phosphorylation (Perez-Pe *et al.* 2002). In this study, we evaluated the effect of BSA

on the capacitation state and protein tyrosine phosphorylation of ram spermatozoa.

A time-dependent increase in the proportion of sperm displaying the capacitation pattern of CTC staining and protein tyrosine phosphorylation occurred in the absence of BSA. After incubation in capacitating conditions, the proportion of CTC capacitation pattern cells increased significantly in a medium with (Fig. 2A) or without (Fig. 2B) BSA, related to the control sample (0 h). The absence of BSA did not affect sperm surviving and a high membrane integrity value was still maintained after 4-h incubation.

The total protein tyrosine phosphorylation signal slightly increased in the course of incubation either with (Fig. 2C) or without BSA (Fig. 2D). The phosphorylation signal of certain faint protein bands of higher (mainly 60–120 molecular region) and lower (<30 kDa) molecular weight slightly increased as incubation

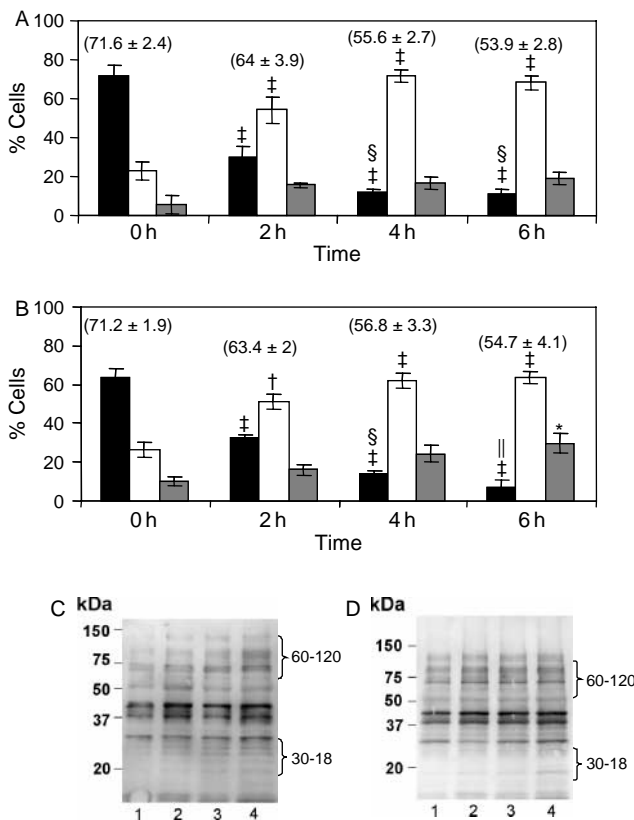


Figure 2 Effect of the incubation time on the capacitation state (CTC/EthD-1 staining) of ram sperm obtained by swim-up and incubated in complete SM (with 3 mM Ca²⁺ and 15 mM HCO₃⁻) at 39 °C under 100% humidity and 5% CO₂. (A) With BSA (5 mg/ml), (B) without BSA. Percentage of ■ not capacitated, □ capacitated, ■ acrosome-reacted sperm. Mean values ± s.e.m. (n=5). In parentheses, sperm membrane integrity is evaluated by carboxyfluorescein diacetate/propidium iodide (CFDA/PI) staining. Significant differences relative to control (0 h): *P<0.05; †P<0.01; ‡P<0.001; or to samples incubated 2 h: §P<0.05; ||P<0.01. Western blot analysis of protein tyrosine phosphorylation (C) with BSA, (D) without BSA. Lanes 1–4: 0, 2, 4, and 6 h incubation respectively. The experiment was performed three times, and a representative membrane is shown.

proceeded. Densitometric analyses confirmed increase percentages in the signalling at 60–120 kDa region of 17.49 ± 7.43, 33.01 ± 4.88 and 48.64 ± 6.27, following 2-, 4- and 6-h incubation with BSA respectively; and 18.22 ± 10.29, 37.07 ± 12.58 and 46.104 ± 19.8, after 2-, 4- and 6-h incubation without BSA respectively. The increases after 6 h were significantly higher (P<0.05) than those found after 2 h in both cases. Similar progressive augmentation was observed at the molecular region <30 kDa with a medium value of 42.55 ± 11.36 and 32.54 ± 6.69 after 6-h incubation with/without BSA respectively.

The addition of increasing concentration of cholesterol sulphate to samples containing BSA did not influence results (Fig. 3). No significant differences were found either in the CTC patterns (Fig. 3A) or protein tyrosine phosphorylation signalling (Fig. 3B).

These data confirm our earlier results that show a progressive increase in tyrosine phosphorylation of sperm proteins over the course of capacitation (Perez-Pe et al. 2002) and demonstrate that BSA is not essential for *in vitro* capacitation like-changes of ram spermatozoa.

Effect of sodium bicarbonate

To determine the effect of NaHCO₃ on sperm capacitation in ram, we incubated sperm samples in capacitating

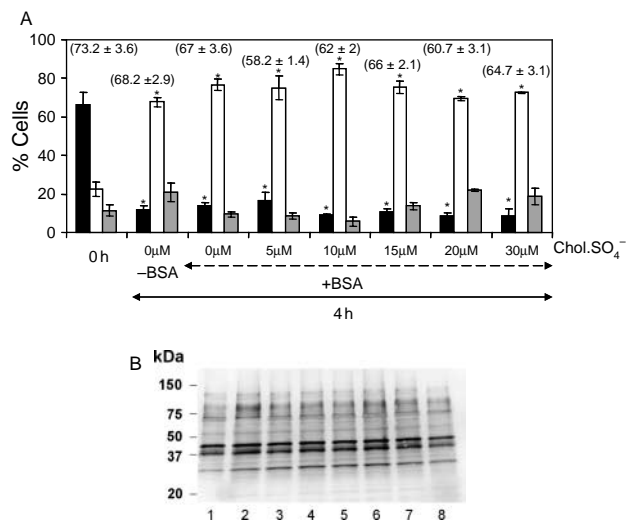


Figure 3 Effect of different cholesterol sulphate concentration on capacitation of ram sperm selected by swim-up and incubated with complete SM (with 3 mM Ca²⁺ and 15 mM HCO₃⁻) at 39 °C under 100% humidity and 5% CO₂. (A) Capacitation state by CTC/EthD-1 staining. Percentage of ■ not capacitated, □ capacitated, ■ acrosome-reacted sperm. Mean values ± s.e.m. (n=3). Significant differences relative to control at 0 h: *P<0.001. In parentheses, sperm membrane integrity evaluated by CFDA/PI staining. (B) Western blot analysis of phosphotyrosine containing proteins. Control samples at 0 h (lane 1); at 4 h without BSA (lane 2) and with BSA (lane 3); capacitated samples in complete SM with BSA and addition of 5, 10, 15, 20 and 30 µM chol - SO₄⁻ (lanes 4–8 respectively). The experiment was performed three times, and a representative membrane is shown.

conditions using a medium containing calcium, either with or without BSA, adding increasing NaHCO_3 concentrations. The proportion of live sperm displaying the capacitation pattern of CTC staining, increased significantly after 4 h incubation in the presence or absence of NaHCO_3 , with (Fig. 4A) or without (Fig. 4B) BSA. It is worth pointing out that a significantly lowered value of sperm displaying the uncapacitated CTC pattern was found in samples with 18 ($P < 0.05$) or 25 mM ($P < 0.01$). Moreover, addition of 25 mM NaHCO_3 accounted for a significant increase in acrosome-reacted sperm, compared with the NaHCO_3 -free control samples. Sperm viability was not significantly affected

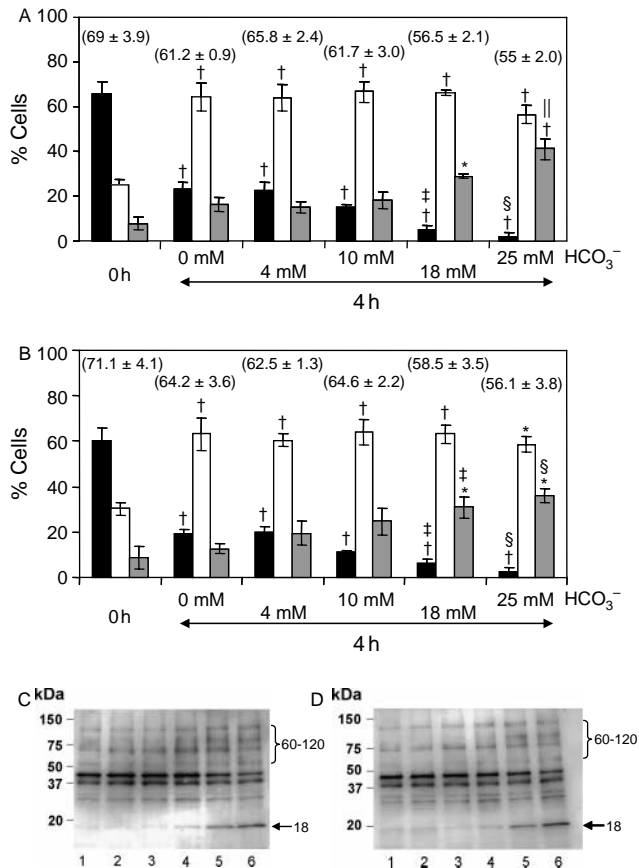


Figure 4 Effect of different HCO_3^- concentrations, in the presence or absence of BSA, on capacitation of ram sperm selected by swim-up and incubated in a medium with 3 mM Ca^{2+} and with increasing concentrations of sodium bicarbonate, at 39 °C under 100% humidity and 5% CO_2 . Capacitation state by the CTC/EthD-1 staining in the presence (A) or absence (B) of BSA (55 mg/ml). Percentage of ■ not capacitated, □ capacitated, ▨ acrosome-reacted sperm. Mean values \pm s.e.m. ($n=4$). Significant differences relative to control at 0 h: * $P < 0.01$; † $P < 0.001$; or to HCO_3^- -free control at 4 h: ‡ $P < 0.05$; § $P < 0.01$; ¶ $P < 0.001$. In parentheses, sperm membrane integrity evaluated by CFDA/PI staining. Western blot analysis of phosphotyrosine containing proteins of samples incubated in the presence (C) or absence (D) of BSA. Lane 1, control sample at 0 h; Lanes 2–6: incubated samples (4 h) with 0, 4, 10, 18 and 25 mM HCO_3^- respectively. The experiments were repeated three times, and a representative membrane is shown.

by the addition of NaHCO_3 up to 18 mM, although it was decreased ($P < 0.05$) at a higher concentration.

Protein tyrosine phosphorylation patterns (Fig. 4C and D) revealed a new protein band of approximately 18 kDa (p18) and a slight increase in some protein bands at the high molecular weight (60–120 kDa) in the presence of bicarbonate. Densitometric analyses confirmed a significant increase ($P < 0.05$) in phosphorylation signalling with 18 or 25 mM bicarbonate, relative to 4 mM bicarbonate containing-sample.

Contribution of extracellular calcium

We studied the effect of adding different Ca^{2+} concentrations to the capacitation medium containing NaHCO_3 . The proportion of live sperm displaying the capacitation pattern of CTC staining (Fig. 5A) did not differ significantly between samples incubated 4 h with or without the addition of calcium. However, a significantly higher ($P < 0.05$) value of acrosome-reacted sperm was found in samples containing 4 mM Ca^{2+} as well as a significantly lower ($P < 0.05$) proportion of not-capacitated sperm in the presence of 2, 3 or 4 mM calcium. Differences in sperm viability were not found in all studied conditions. Similarly, the presence of calcium in the incubation medium did not significantly promote protein tyrosine phosphorylation. A similar pattern of

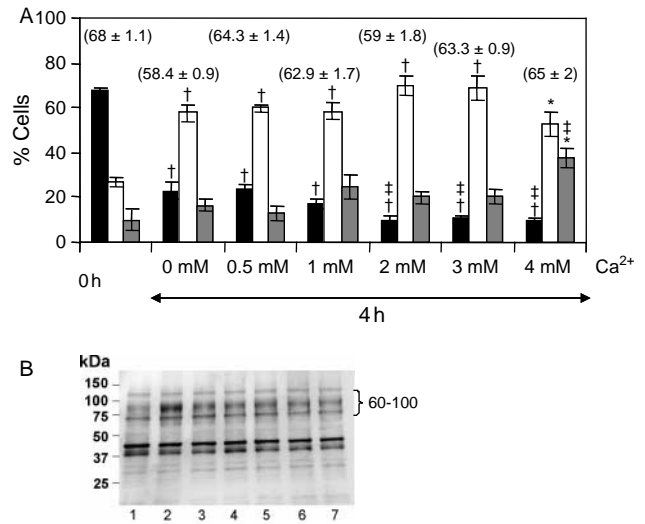


Figure 5 Effect of calcium on capacitation of ram sperm selected by swim-up and incubated at 39 °C under 100% humidity and 5% CO_2 in SM with 15 mM HCO_3^- . (A) Capacitation state by the CTC/EthD-1 staining. Percentage of ■ not capacitated, □ capacitated, ▨ acrosome-reacted sperm. Mean values \pm s.e.m. ($n=4$). Significant differences relative to control at 0 h: * $P < 0.01$; † $P < 0.001$; or to calcium-free control at 4 h: ‡ $P < 0.05$. In parentheses, sperm membrane integrity evaluated by CFDA/PI staining. (B) Western blot analysis of phosphotyrosine containing proteins. Lane 1: control (0 h); lane 2: calcium-free control sample containing 6 mM EGTA at 4 h; lanes 3–7: incubated samples (4 h) with 0.5, 1, 2, 3 and 4 mM Ca^{2+} respectively. The experiment was repeated three times, and a representative membrane is shown.

tyrosine-phosphorylated protein bands was found in all samples after 4 h incubation (Fig. 5B). Moreover, a slight increase in the signalling at the high molecular weight region (60–100 kDa) was observed without the addition of calcium.

Effect of cAMP-elevating agents

We assessed the effect of cAMP-elevating agents (dbcAMP and IBMX) on the capacitation state and phosphorylation patterns of ram sperm samples containing BSA with or without calcium and bicarbonate. The capacitation pattern analysis (CTC/EthD1 staining) revealed that the addition of dbcAMP and IBMX to samples without calcium or bicarbonate, produced a significant increase in sperm displaying CTC capacitation pattern (along with a decrease in not-capacitated pattern) relative to control samples at 0 ($P < 0.001$) or 4 h incubation ($P < 0.01$ and $P < 0.001$), at both assayed concentrations (Fig. 6A). In addition, in the presence of calcium and bicarbonate, a significantly higher value of the acrosome-reacted population was found at the highest concentration of both agents (Fig. 6B). Likewise, in these conditions, a new tyrosine-phosphorylated protein band, approximately 100 kDa was detected together with a slight increase in the labelling of p30 and at the 60–120 kDa molecular region (Fig. 6C and D). The increase in the phosphorylation signalling after 4 h incubation with the highest concentration of dbcAMP and IBMX related to the control sample (incubated 4 h without effectors) was $26.02\% \pm 2.0$ in the absence and $17.97\% \pm 6.3$ in the presence of calcium and bicarbonate. Moreover, the phosphorylation labelling obtained after 4 h incubation compared with signal at 0 h was significantly higher ($P < 0.05$) in samples containing dbcAMP and IBMX than in the free-effector control samples.

Effect of PKA inhibitor and calcium entry blocker on capacitation and protein phosphorylation

To determine whether ram sperm capacitation and protein phosphorylation is regulated through a cAMP/PKA pathway, we assessed the capacitation state of ram spermatozoa incubated in a complete medium with/without different concentrations of a PKA inhibitor (H-89, 10–200 μM), or with an antagonist of calcium channels (Verapamil, 0.05–1 mM).

The CTC pattern obtained after 4 h incubation in capacitating conditions with H-89 (Fig. 7A) showed a significant decrease ($P < 0.05$) in sperm displaying the CTC-capacitated pattern in the presence of 200 μM H-89 relative to control (4 h). A slight decrease in tyrosine-phosphorylated proteins at the 60–120 kDa molecular region was observed (Fig. 7C). Densitometric analysis revealed a decrease in the phosphorylation

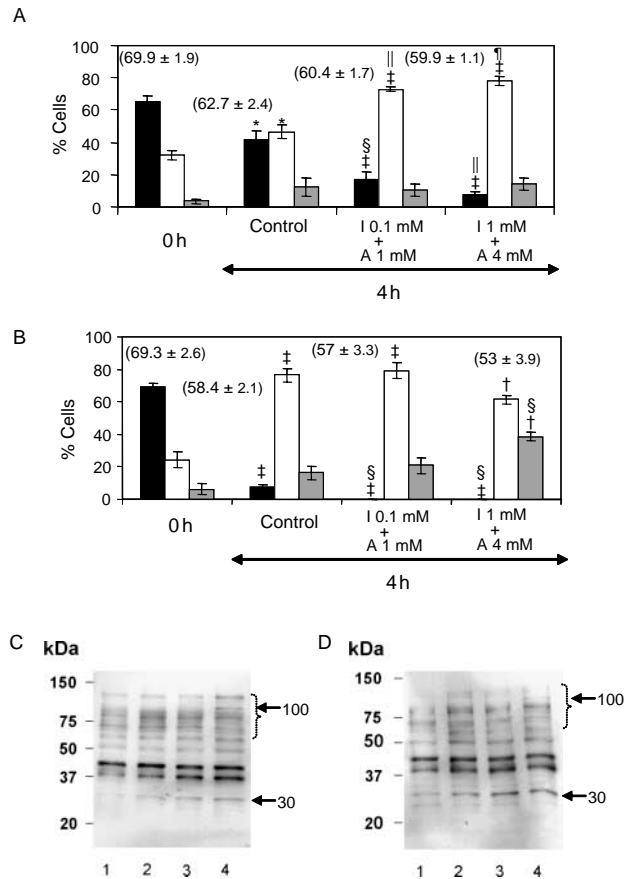


Figure 6 Effect of IBMX (a non specific phosphodiesterase inhibitor) and db-cAMP (an analogue of cAMP) on the capacitation state and phosphotyrosine containing proteins profile. Assessment of the capacitation state by the CTC/EthD-1 staining of sperm selected by swim-up incubated 4 h at 39 °C under 100% humidity and 5% CO₂, in the presence of BSA (5 mg/ml): A) in a calcium and bicarbonate-free medium with 6 mM EGTA; B) in a medium with 3 mM Ca²⁺ and 15 mM HCO₃⁻. IBMX (I, 0.1 and 1 mM) and db-cAMP (A, 1 and 4 mM) were added to sperm samples. Percentage of ■ not capacitated, □ capacitated, ▒ acrosome-reacted sperm. Mean values ± s.e.m. (n = 4). Significant differences relative to control at 0 h: *P < 0.05; †P < 0.01; ‡P < 0.001; or to drugs-free control at 4 h: §P < 0.05; ¶P < 0.01; ††P < 0.001. In parentheses, sperm membrane integrity evaluated by CFDA/PI staining. Western blot analysis of phosphotyrosine containing proteins: (C) in a calcium and bicarbonate-free medium with 6 mM EGTA; (D) in a medium with 3 mM Ca²⁺ and 15 mM HCO₃⁻. Lane 1 and 2: control samples at 0 and 4 h incubation respectively; lane 3: capacitated sample with 0.1 mM IBMX and 1 mM db-cAMP; lane 4: capacitated samples with 1 mM IBMX and 4 mM db-cAMP. The experiment was repeated three times and a representative membrane is shown.

signal relative to control sample after 4 h. The assessed percentages of decrease were 10.59 ± 0.258 , 16.17 ± 3.49 and 29.45 ± 2.65 with 50, 100 and 200 μM H-89 respectively. Of these, the percent decrease obtained with 200 μM H-89 was significantly greater than those with 50 and 100 μM ($P < 0.01$ and $P < 0.05$ respectively). In contrast, no significant differences were found either in CTC patterns (Fig. 7B) or in phosphorylation signalling (Fig. 7D) between samples with or without Verapamil after 4 h incubation.

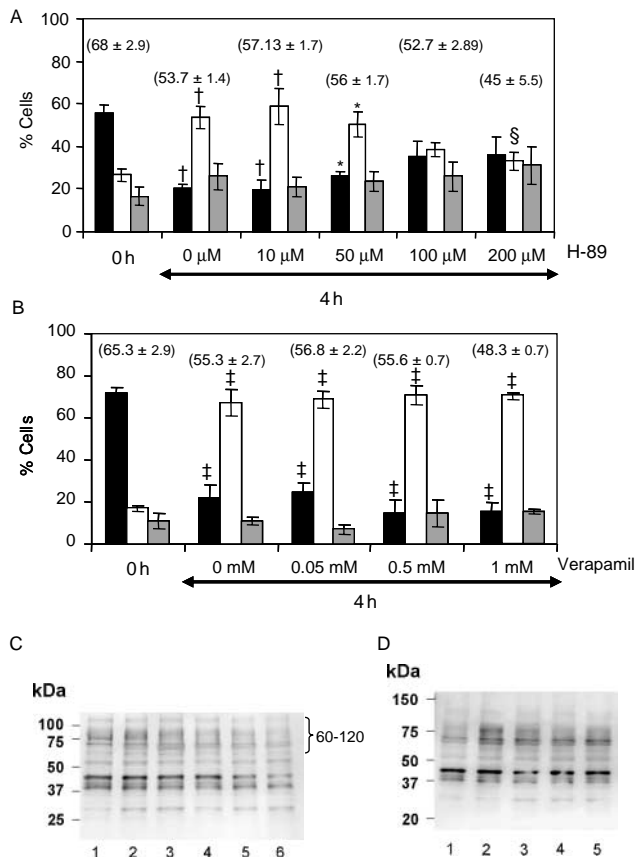


Figure 7 Effect of a calcium entry blocker (Verapamil, 0.05, 0.5 and 1 mM) and a PKA inhibitor (H-89, 10, 50 100 and 200 μ M) on capacitation of ram sperm selected by swim-up and incubated at 39 °C under 100% humidity and 5% CO₂ in a complete medium (with 3 mM Ca²⁺ and 15 mM HCO₃⁻) in the presence of BSA (55 mg/ml). Capacitation state assessed by the CTC/EthD-1 staining (A) in the presence of H-89, (B) in the presence of Verapamil. Percentage of ■ not capacitated, □ capacitated, ■ acrosome-reacted sperm. Mean values \pm S.E.M. ($n=4$). Significant differences relative to control at 0 h: * $P<0.05$, † $P<0.01$, ‡ $P<0.001$; or to drug-free control at 4 h: § $P<0.05$. In parentheses, sperm membrane integrity evaluated by CFDA/PI staining. Western blot analysis of phosphotyrosine containing proteins: (C) samples treated with H-89: Lanes 1 and 2: control (0 h) and capacitated samples (4 h); Lanes 3, 4, 5 and 6: capacitated samples in the presence of 10, 50 100 and 200 μ M H-89 respectively. (D) Samples treated with Verapamil: Lanes 1 and 2: control (0 h) and capacitated samples (4 h); Lanes 3, 4, and 5: capacitated samples in the presence of 0.05, 0.5 and 1 mM Verapamil respectively. The experiment was performed three times, and a representative membrane is shown.

Discussion

Although the CTC assay has been used to assess capacitation-like changes in ram spermatozoa (Perez *et al.* 1996, Gillan *et al.* 1997, Perez-Pe *et al.* 2002), in this study, we designed an initial assay to validate the CTC technique. The double estimation using FITC-RCA/EthD-1 and CTC/EthD-1 labelling showed logical data after incubation in capacitating conditions and highly consistent results were obtained after *in vitro* induction of the acrosome reaction by LPC, which produced a

significant increase in acrosome-reacted sperm, as already shown in ram (Gomez *et al.* 1997) and bull (Cormier & Bailey 2003) sperm. Moreover, we found a positive correlation between the results of acrosome-reacted sperm evaluated with both techniques, RCA (shown to accurately assess the acrosomal status of ram spermatozoa (Marti *et al.* 2000) and CTC. These data indicate that CTC staining in combination with EthD-1 can be useful for the assessment of ram sperm capacitation state in standard conditions. Although compounds that alter membrane fluidity may change CTC patterns (Tardif *et al.* 1999), we can suggest that the changes on CTC patterns obtained after incubation in different conditions (time of incubation, bicarbonate and cAMP-elevating agents) could correlate with capacitation-based on the validation assay performed in this study.

Results of this study demonstrate that, in ejaculated ram spermatozoa, there is a time-dependent increase in capacitation and the increase is, at least, partially correlated with protein tyrosine phosphorylation, in either the presence or absence of BSA (Fig. 2). In this study, to enhance the immunodetection resolution, we increased the number of sperm/sample (10^7) compared with our previous study (Perez-Pe *et al.* 2002), which increased the amount of extracted proteins. Therefore, the amount of protein analysed by western blotting was higher and the assay sensitivity was increased. In these conditions, tyrosine phosphorylation of p30 was detectable in control samples (0 h; Fig. 2C and D), whereas, in the earlier study, we only detected this phosphorylated protein band after *in vitro* capacitation. Since 0 h capacitation was preceded by 1 h swim-up procedure, the phosphorylation of proteins might have started during the swim-up process. In addition, results with boar sperm showed that protein phosphorylation can occur sometime during and before the completion of capacitation and once phosphorylated, the apparent level of protein phosphorylation remains relatively constant (Tardif *et al.* 2001). However, we confirmed a significantly higher proportion of not capacitated and viable spermatozoa in the swim-up obtained sample, compared with another three washing methods (sucrose centrifugation, Percoll gradient and filtration), while the protein tyrosine phosphorylation pattern was very similar in all obtained samples (data not shown).

Our results show that the presence of BSA does not affect ram sperm changes induced by incubation in capacitating conditions. BSA promotes capacitation by removing cholesterol from the sperm plasma membrane, which reduces the cholesterol:phospholipids (C:P) ratio and increases membrane fluidity (Davis 1981, Zarintash & Cross 1996). The decrease in cholesterol could activate PKA and protein tyrosine phosphorylation as it has been speculated (Visconti *et al.* 1995a); however, the C:P ratio of the plasma membrane of ram sperm (0.38) is considerably lower than in some other species (Parks &

Hammerstedt 1985), including human (0.99; Darin-Bennett & White 1977). Therefore, due to the low C:P ratio, albumin-mediated cholesterol efflux might not be necessary during ram sperm capacitation, which is indicated by the apparent irrelevance of BSA to *in vitro* capacitation and associated protein phosphorylation. These findings are similar to that reported for boar sperm (Tardif *et al.* 2003) also with a very low C:P ratio (Parks & Lynch 1992). Furthermore, the addition of cholesterol up to 30 μM , able to inhibit capacitation and protein tyrosine phosphorylation in mouse (Visconti *et al.* 1999a) and human (Osheroff *et al.* 1999), did not influence results (Fig. 3). However, it was recently demonstrated in boar sperm (using calcium ionophore-induced AR and protein tyrosine phosphorylation) that although cholesterol efflux does not appear to be necessary for boar sperm capacitation, it could be completely inhibited by incubation with hydroxyl- β -cyclodextrin plus cholesterol sulphate. The cyclodextrin delivers the cholesterol sulphate to the plasma membrane and increases the C:P ratio blocking capacitation (Galantino-Homer *et al.* 2006). Further investigations to prove this effect in ram sperm have to be done, as the concentration of cholesterol sulphate that inhibited boar sperm capacitation was much higher than that used in our study.

In several species, bicarbonate is a requirement for *in vitro* capacitation (Shi & Roldan 1995, Visconti *et al.* 1995a, 1999b, Carrera *et al.* 1996, Tardif *et al.* 2003), and is a key regulator of protein tyrosine phosphorylation in mature mammalian spermatozoa (Visconti *et al.* 1995a, Aitken *et al.* 1998). The role of NaHCO_3 in capacitation is probably mediated by its ability to increase intracellular pH (Zeng *et al.* 1996) and stimulate adenylate cyclase (Okamura *et al.* 1985, Visconti *et al.* 1990), as well as its ability to modify the lipid architecture of plasma membranes (Harrison & Gadella 1995, 2005, Gadella & Harrison 2000). Here, we investigated whether bicarbonate, alone or in combination with BSA, influences ram sperm capacitation. The addition of high bicarbonate concentrations (18 and 25 mM) did result in a significant decrease in not capacitated spermatozoa with respect to free-bicarbonate control samples, together with a significantly higher proportion of acrosome-reacted cells (Fig. 4A). These changes in CTC patterns were parallel to an increase in protein tyrosine phosphorylation signalling (Fig. 4C and D). These results are consistent with those reported in mouse (Shi & Roldan 1995, Visconti *et al.* 1995a), boar (Harrison *et al.* 1996, Tardif *et al.* 2003) and human (Carrera *et al.* 1996). Nevertheless, the capacitation-associated protein tyrosine phosphorylation was shown not to require bicarbonate in hamster (Visconti *et al.* 1999b, Kulanand & Shivaji 2001) and boar (Tardif *et al.* 2003).

The essential role of extracellular calcium in mammalian fertilization is commonly accepted and empirically well established (Yanagimachi 1994). Studies have

demonstrated that extracellular calcium influences capacitation in several mammalian species (Visconti *et al.* 1995a, Luconi *et al.* 1996, Tardif *et al.* 2001) and modulates enzymes involved in signal transduction, such as adenylate cyclase and cyclic nucleotide phosphodiesterase (Visconti *et al.* 1998). In our study, the addition of a high concentration of extracellular Ca^{2+} to a medium containing bicarbonate did not increase the capacitated subpopulation, but did increase the acrosome-reacted sperm (Fig. 5A). Therefore, the participation of calcium as an inducer of membrane changes needed for the subsequent acrosome reaction cannot be ruled out. However, we found an increase in protein tyrosine phosphorylation signal related to capacitation even in the absence of extracellular calcium which is consistent with that reported in hamster (Kulanand & Shivaji 2001) and indicates that protein tyrosine phosphorylation could be at least partially independent of calcium. In this sense, there are controversial published results about the effect of extracellular calcium on *in vitro* sperm capacitation and protein tyrosine phosphorylation depending on species and/or laboratories (Visconti *et al.* 1995a, Luconi *et al.* 1996, Tardif *et al.* 2003, Baker *et al.* 2004). This controversy might be due to differences in species-specific reproduction strategies (Kalab *et al.* 1998).

The interdependent roles of calcium and bicarbonate in mammalian sperm capacitation can be deduced from their involvement in the regulation of cAMP levels (Visconti *et al.* 1995a). The levels of intracellular cAMP are regulated by the activity of two enzymes that are implicated in the generation (adenylate cyclase, AC) and degradation (phosphodiesterase, PDE) of cAMP. Bicarbonate stimulates AC activity and transiently increases cAMP levels which favours protein tyrosine phosphorylation during capacitation (Okamura *et al.* 1985, Harrison & Miller 2000). Visconti *et al.* (1998) proposed a working model for the regulation of sperm capacitation in, which bicarbonate and calcium exert their effects through AC and, therefore, the absence of either of these effectors might not affect the capacitation process. Our results show that ram spermatozoa incubated in the absence of both bicarbonate and calcium (Fig. 6A) are capacitated to a lesser extent than those in the presence of each or both compounds. These findings suggest that Visconti's model might function in ram spermatozoa with an interdependent role of calcium and bicarbonate that might exert their effects through AC, thereby increasing sperm cAMP to levels that favour protein tyrosine phosphorylation during capacitation. Addition of cAMP-elevating agents accounted for a significant increase in capacitated sperm in the absence of calcium and bicarbonate, and the obtained CTC-pattern was nearly the same as that found after 4 h incubation in the presence of bicarbonate and calcium, in the effectors-free control sample. Furthermore, protein phosphorylation was enhanced in both

conditions (Fig. 6C and D) and a new protein band of approximately 100 kDa was found. These results suggest that cAMP and IBMX might bypass the requirement for bicarbonate and calcium in ram sperm capacitation.

Although, several studies have implicated PKA and cAMP in the regulation of protein tyrosine phosphorylation during capacitation, the molecular basis of the process is poorly understood (Visconti *et al.* 1995b, 1999b, Leclerc *et al.* 1996, Galantino-Homer *et al.* 1997, Kalab *et al.* 1998). It is worth noting that although we confirm that protein tyrosine phosphorylation in ram sperm is induced by treatments expected to up-regulate cAMP, tyrosine phosphorylation of at least certain protein bands appears to be independent of such induced changes in cAMP levels. These results suggest there might be certain phosphorylated proteins that are not influenced by the elevation of cAMP levels, as already described in goat (Maiti *et al.* 2004) and boar (Kalab *et al.* 1998, Tardif *et al.* 2004). The contribution of other pathway involving receptor of tyrosine kinases and non-receptor protein tyrosine kinases cannot be ruled out, as already proposed (Naz & Rajesh 2004).

Moreover, our results show that addition of cAMP-elevating agents induced an increase in acrosome-reacted sperm after 4 h incubation in capacitating conditions. Similarly, an increase in induced acrosome reactions by LPC in bovine (Parrish *et al.* 1994) and by calcium ionophore in human (Ford *et al.* 1994) and ram (Garde & Roldan 2000) sperm, has been described in response to an increase in cAMP levels. Likewise, the involvement of cAMP-PKA pathway in acrosome reaction (Breitbart & Naor 1999, Garde & Roldan 2000), and the activation of calcium channels involved in acrosome reaction by the increase in cAMP-PKA activity during capacitation has also been reported (Breitbart 2002).

To confirm the implication of the PKA/AMPC pathway in sperm capacitation in ram, we assessed the effect of PKA inhibition. Our results (Fig. 7) show that H-89, a relatively specific PKA inhibitor (Chijiwa *et al.* 1990), caused a significant decrease in sperm capacitation in concordance to previous reports in mouse (Visconti *et al.* 1995b) and boar (Tardif *et al.* 2004). Moreover, the protein tyrosine phosphorylation signalling of a subset of proteins of high molecular weight was slightly lowered, consistent with that reported in other species (Visconti *et al.* 1995b, Galantino-Homer *et al.* 1997, Pommer *et al.* 2003). However, the concentration of H-89, able to significantly inhibit ram sperm capacitation is higher than that found in boar (Tardif *et al.* 2004) suggesting, once more, a specie-specific susceptibility to cAMP modulation.

Conversely, the addition of Verapamil did not significantly influence either capacitation or protein tyrosine phosphorylation, which suggests that the calcium entry blocker was not able to inhibit the calcium influx associated to capacitation in ram sperm.

Similar results have been described in bull sperm in which Verapamil up to 0.75 mM did not affect calcium uptake (Breitbart & Lardy 1987) and mouse sperm where calcium channel antagonists influence calcium movements at the end of capacitation, but not during earlier phases of the process (Fraser & McIntyre 1989).

Here, we have presented basic information on the signal transduction pathways that are activated during capacitation of ram spermatozoa. Our results demonstrate that in ram sperm, capacitation and the slight associated protein tyrosine phosphorylation is not absolutely dependent on the presence of BSA and calcium, and the PKA/cAMP pathway is, at least, partially implicated in the tyrosine phosphorylation of some proteins.

The increase in protein tyrosine phosphorylation observed in our study, even in the absence of calcium, suggests that these events are not an end point of sperm capacitation in ram. These phosphorylations should not be considered strictly indicative of full capacitation in ram sperm, as described in human (Emiliozzi & Fenichel 1997) and boar (Tardif *et al.* 2003), where protein tyrosine phosphorylation alone was not sufficient for the completion of capacitation. These data indicate that the signal transduction mechanisms of capacitation in ram sperm differ from those in other mammals, which suggests that species specificities might exist with respect to this process.

Further studies are necessary to determine the specific molecules that undergo differential phosphorylation at tyrosine residues during capacitation that leads to signal transduction downstream to induce the acrosome reaction. This might have implications with regard to male fertility diagnosis.

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