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.


Introduction


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.


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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 U/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 Spillman 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 osmolality 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 (cholesterol-3-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-methylxantine (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 (10⁶ 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 carboxylfluorescein 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 ± S.E.M.

We used a modified version of the chlorotetracycline (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 µM, Sigma) was prepared daily in a buffer containing 20 mM Tris, 130 mM NaCl and 5 µM cysteine, pH 7.8, and passed through a 0.22 µm filter (Millipore Ibérica, Madrid, Spain). EthD-1, 2 µl (23.3 µM) were added to 18 µl sperm sample, and incubated at 37 °C in the dark for 10 min. Thereafter, 20 µl CTC solution and 5 µl of 12.2% (w/v) paraformaldehyde in 0.5 M Tris–HCl, pH 7.8, were added. At room temperature, a 4 µl aliquot of stained sample was placed on a glass slide and mixed with 2 µl 0.22 M triethylene diamine (DABCO, Sigma) in 9 glycerol:1 PBS. The samples were covered with 24×48 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 µM) 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 (107 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 µl extraction medium (2% SDS, 28% sucrose, 12.4 mM Tris–HCl, pH 6.8, 0.4 mM tetrazolium, 5 mM MgCl2, 0.38 mM phosphate) and incubated at 37 °C for 10 min. Thereafter, 20 µl CTC solution and 5 µl of 12.2% (w/v) paraformaldehyde in 0.5 M Tris–HCl, pH 7.8, were added. At room temperature, a 4 µl aliquot of stained sample was placed on a glass slide and mixed with 2 µl 0.22 M triethylene diamine (DABCO, Sigma) in 9 glycerol:1 PBS. The samples were covered with 24×48 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.

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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 (1×107 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 IgG (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

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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 x intensity), and changes relative to control (sample without effector incubated 4 h) are presented as a percentage.

**Statistical analysis**

Results are presented as mean ± 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 (Saleng & 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 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 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 46.10 ± 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...
conditions using a medium containing calcium, either with or without BSA, adding increasing NaHCO₃ 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 NaHCO₃ 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 NaHCO₃ accounted for a significant increase in acrosome-reacted sperm, compared with the NaHCO₃-free control samples. Sperm viability was not significantly affected by the addition of NaHCO₃ 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 Ca²⁺ concentrations to the capacitation medium containing NaHCO₃. 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 Ca²⁺ 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 4](image_url)

**Figure 4** Effect of different HCO₃⁻ 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 Ca²⁺ and with increasing concentrations of sodium bicarbonate, at 39 °C under 100% humidity and 5% CO₂. 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 ± s.e.m. (n=4). Significant differences relative to control at 0 h: *P<0.01; §P<0.001; or to HCO₃⁻-free control at 4 h: †P<0.05; ‡P<0.01. 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 HCO₃⁻ respectively. The experiments were repeated three times, and a representative membrane is shown.

![Figure 5](image_url)

**Figure 5** Effect of calcium on capacitation of ram sperm selected by swim-up and incubated at 39 °C under 100% humidity and 5% CO₂ in SM with 15 mM HCO₃⁻. (A) Capacitation state by the CTC/EthD-1 staining. Percentage of □ not capacitated, □ capacitated, □ acrosome-reacted sperm. Mean values ± s.e.m. (n=4). Significant differences relative to control at 0 h: *P<0.01; §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 Ca²⁺ 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%±2.0 in the absence and 17.97%±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 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.
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 (Martí 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 (10⁷) 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 NaHCO₃ in capacitation is probably mediated by its ability to increase intracellular pH (Zeng et al. 1996) and stimulate adenylyl 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 adenylyl cyclase and cyclic nucleotide phosphodiesterase (Visconti et al. 1998). In our study, the addition of a high concentration of extracellular Ca²⁺ 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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