Changes in content and localization of proteins phosphorylated at tyrosine, serine and threonine residues during ram sperm capacitation and acrosome reaction

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Abstract

Previously, we reported the involvement of tyrosine phosphorylation in events that lead to ram sperm capacitation. In this study, we carried out a comparative analysis of the localization of tyrosine, serine and threonine phosphoproteins in different functional stages of ram spermatozoa (after the swim-up procedure, *in vitro* capacitation, and ionophore-induced acrosome reaction) by immunofluorescence, immunocytochemistry and confocal microscopy. Capacitation increased protein tyrosine, serine and threonine phosphorylation whereas the induction of the acrosome reaction resulted in significantly decreased phosphorylation, mainly in those proteins that increased following capacitation. Control samples showed tyrosine-phosphorylated proteins restricted to the head, mainly distributed at the equatorial region with some cells also displaying an acrosomal and/or post-acrosomal localization. *In vitro* capacitation promoted both tail and acrosome phosphorylation, and the acrosome reaction induced the loss of labeling on the acrosome and the subsequent increase in the post-acrosomal region and flagellum. The preferential localization of serine- and threonine-phosphorylated proteins in the equatorial and acrosomal regions found in control samples changed during capacitation, which induced tail phosphorylation in a sequential manner. After the acrosome reaction, the labeling of both phosphoamino acids decreased in the acrosome and increased in the post-acrosome. The obtained results were proved by two immunodetection techniques and strengthened by confocal microscopy, and indicate that changes in phosphorylated proteins during capacitation and acrosome reaction of ram spermatozoa may have physiological significance in consolidating certain phosphorylated proteins to specific sperm regions involved in acrosomal exocytosis and zona pellucida recognition, binding and penetration.

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Introduction

After ejaculation, mammalian spermatozoa are unable to fertilize the egg. They become gradually fertilization competent after a complex set of modifications during a finite period of residence in the female reproductive tract. During this time, the spermatozoa undergo a remodeling process known as capacitation, which is characterized by a series of profound biochemical and biophysical transformations by which the spermatozoa acquire the fertilizing ability (Yanagimachi 1994). The molecular events associated with capacitation are involved in the induction of hyperactivation, characterized by changes in sperm motility patterns (Yanagimachi 1994, Suarez 1996) and acrosome reaction (Visconti *et al.* 2002). The acrosome reaction is a specialized exocytotic event consisting of the

fenestration and fusion of the outer acrosomal membrane and the overlying plasma membrane at multiple sites (Yanagimachi 1994), which results in both the release of hydrolytic enzymes and in the exposure of new membrane domains, both of which are essential for the fertilization process. These two processes, capacitation and acrosome reaction, are regulated by intracellular signals and associated with protein phosphorylation (Aitken *et al.* 1995, Burks *et al.* 1995, Baldi *et al.* 1996, Visconti & Kopf 1998, Visconti *et al.* 2002, Naz & Rajesh 2004, Liguori *et al.* 2005, O'Flaherty *et al.* 2006a). However, the precise mechanisms involved in sperm capacitation and acrosomal exocytosis are still not fully understood.

Protein phosphorylation is a post-translational modification of proteins that allows the cell to control many

cellular processes such as transduction of extracellular signals and intracellular transport (Ullrich & Schlessinger 1990, Johnson & Barford 1993, Johnson & O'Reilly 1996). Mature spermatozoa are highly specialized cells with specific functions directed to fertilization that are compartmentalized within specific subcellular regions. Post-translational protein phosphorylation plays a key role in sperm function and regulates processes such as capacitation, hyperactivation motility and the acrosome reaction. Signal transduction involves reversible phosphorylations regulated through protein kinases and phosphatases, which occurs predominantly on serine, threonine and tyrosine residues (Johnson & Barford 1993, Wera & Hemmings 1995). However, to date, only a few sperm-phosphorylated proteins have been identified and the knowledge of sperm tyrosine kinases and other serine/threonine kinases remains limited.

In most species studied, sperm tyrosine-phosphorylated proteins are mainly located at the flagellum during the course of capacitation and fertilization (Carrera *et al.* 1996, Leclerc *et al.* 1997, Mahony & Gwathmey 1999, Si & Okuno 1999, Lewis & Aitken 2001, Urner *et al.* 2001, Petrunkina *et al.* 2003, Pommer *et al.* 2003, Sakkas *et al.* 2003), and the presence of tyrosine phosphorylation in the principal piece has been correlated with the acquisition of hyperactivated motility (Nassar *et al.* 1999, Si & Okuno 1999, Petrunkina *et al.* 2003). In addition, a capacitation-associated redistribution of phosphotyrosine residues to the acrosome has been reported in boars (Petrunkina *et al.* 2001, Tardif *et al.* 2001), bulls (Cormier & Bailey 2003) and buffalo (Roy & Atreja 2008), and specific phosphotyrosine proteins localized over the acrosomal region have been postulated to be involved in the zona pellucida interaction and/or fusion events (Leyton & Saling 1989, Naz *et al.* 1991, Ficarro *et al.* 2003, Dube *et al.* 2005).

Although very little is known about serine/threonine phosphorylation, several studies have shown a time-dependent increase in serine and/or threonine phosphorylation during sperm capacitation in species such as humans (Naz 1999, O'Flaherty *et al.* 2004), hamsters (Jha & Shivaji 2002b), boars (Harayama 2003) and mice (Jha *et al.* 2006). Besides the well-characterized protein kinase A (PKA), other Ser/Thr kinases and the ERKs have been involved in sperm capacitation and acrosome reaction (Luconi *et al.* 1998, du Plessis *et al.* 2001, de Lamirande & Gagnon 2002, Liguori *et al.* 2005). More recently, specific targets to PKA phosphorylation has been established in human (O'Flaherty *et al.* 2004, Moseley *et al.* 2005) and boar sperm (Harrison 2004). Likewise, a cAMP-independent serine/threonine phosphorylation has been described. Thus, an ecto-cAMP-independent protein kinase has been located on the outer surface of mature goat spermatozoa (Maiti *et al.* 2004) and a proline-directed serine/threonine phosphorylation associated to mouse sperm capacitation has been reported (Jha *et al.* 2006).

In a previous report, we demonstrated an increase in protein tyrosine phosphorylation associated to capacitation in ram spermatozoa (Perez-Pe *et al.* 2002), and that the cAMP-PKA pathway is partially implicated in ram sperm capacitation (Grasa *et al.* 2006). Therefore, our first objective was to determine whether serine and threonine phosphorylation is involved in events that lead to ram sperm capacitation. In addition, we also investigated the protein phosphorylation associated to the acrosome reaction induced by calcium ionophore A23187. Furthermore, we analyzed the localization of serine, threonine and tyrosine phosphoproteins associated to different functional stages of ram spermatozoa (after the swim-up procedure, *in vitro* capacitation, and acrosome reaction) by means of immunocytochemical techniques.

Results

Protein phosphorylation associated with *in vitro* capacitation and acrosome reaction

In a previous study, we reported that the incubation of fresh ram spermatozoa in capacitating conditions is associated to an increase in protein tyrosine phosphorylation (Grasa *et al.* 2006). In this study, we investigated whether ram sperm capacitation induced *in vitro* is related to serine/threonine phosphorylation, as well as the profile of phosphorylated proteins associated to the ionophore-induced acrosome reaction. Evaluation of the physiological state by chlortetracycline (CTC)/ethidium homodimer 1 (EthD-1) staining (Fig. 1) showed a significant increase ($P < 0.001$) in the percentage of capacitated sperm after 4 h of incubation in capacitating conditions, compared with control samples (0 h). Likewise, a significant increase ($P < 0.001$) in the acrosome-reacted (AR) CTC-pattern was achieved in samples stimulated with calcium ionophore (Fig. 1).

Figure 2 shows the phosphoprotein profile of control, capacitated and AR samples. Several proteins were

Figure 1 Assessment of the capacitation state by CTC/EthD-1 staining of ram spermatozoa obtained by swim-up at 0 h (control samples), incubated 4 h in capacitation conditions at 39 °C under 100% humidity and 5% CO₂ (capacitated samples), and treated with 3 µM calcium ionophore A23187 for 1 h at 39 °C (acrosome-reacted samples). Percentage of cells: black bars, not capacitated; white bars, capacitated; gray bars, acrosome reacted. Mean values $\pm$ S.E.M. ($n = 5$). Significant differences related to control samples: * $P < 0.05$; *** $P < 0.001$. Significant differences related to capacitated samples: ### $P < 0.001$.

Figure 2 Immunoblot analysis of the effect of the capacitation and the A23187-induced acrosome reaction on ram sperm protein phosphorylation in tyrosine (A), serine (B) and threonine (C) residues. Sperm were obtained by swim-up (control samples, lane 1), incubated in capacitation conditions for 4 h at 39 °C under 100% humidity and 5% CO₂ (capacitated samples, lane 2), and treated with 3 µM calcium ionophore A23187 for 1 h at 39 °C (acrosome-reacted samples, lane 3). Binding of antibody in the presence of 100 µg/ml (lane 4) and 500 µg/ml (lane 5) of phosphotyrosine, 25 µg/ml (lane 4) and 50 µg/ml (lane 5) of phosphoserine, and 50 µg/ml (lane 4) and 100 µg/ml (lane 5) of phosphothreonine, after incubation with 23 U alkaline phosphatase (lane 6). Positive (casein, lane 7) and negative (FurA of *Anabaena* sp. PPC7 120, lane 8) controls are shown. Proteins extracted from 10⁷ spermatozoa were analyzed by western blotting, as described under M&M. The experiment was performed three times and representative images are shown.

already phosphorylated at tyrosine, serine and threonine residues in control samples (Fig. 2A–C), as we have already reported at tyrosine (Perez-Pe *et al.* 2002, Grasa *et al.* 2006). We confirmed that *in vitro* capacitation promoted a significant increase in the content of some

bands phosphorylated at tyrosine (Table 1) as reported previously (Grasa *et al.* 2006).

Regarding phosphorylation at serine residues, three molecular weight regions were distinguished in western blots of control samples (Fig. 2B). Three bands at the high molecular mass region (84–120 kDa), four bands at the 40–70 kDa molecular mass region (one thicker of 55 kDa and three of ~70, 45 and 40 kDa), and another two weak bands of low molecular mass (<30 kDa). *In vitro* capacitation promoted a significant increase in the detected bands at the 84–120 kDa and 45–40 kDa molecular mass regions (Table 1).

The anti-phosphothreonine antibody specifically recognized a set of phosphoproteins in control samples (Fig. 2C). At least eight protein bands were detected, two bands in the 84–120 kDa molecular mass region, (a faint band of ~120 kDa and another of 84 kDa), five bands ranged from 40 to 70 kDa molecular masses, and another of ~30 kDa. Densitometric quantification revealed that capacitation resulted in a significantly higher intensity of the bands in these three molecular weight regions (Table 1).

The ionophore-induced acrosome reaction was accompanied by a significant decrease in the intensity of phosphorylation for all three residues (Table 1; Fig. 2, lane 3). It is worth pointing out that certain bands of phosphotyrosine- (84–120, 40–70, and <30 kDa molecular mass regions, Fig. 2A, lane 3), serine- (40–45 kDa and 84–120 kDa molecular mass regions, Fig. 2B, lane 3) and threonine- (84–120, 40–70, and <30 kDa molecular mass regions, Fig. 2C, lane 3) phosphorylated proteins with increased intensity after capacitation showed a significantly lower intensity labeling after the acrosome reaction.

Incubation of membranes with phosphotyrosine, phosphoserine, or phosphothreonine proved the

Table 1 Densitometric analysis of the of phosphorylation signal at tyrosine (Tyr), serine (Ser) and threonine (Thr) residues in control (CT), capacitated (CA) and acrosome-reacted (AR) sperm samples.

	Molecular region	Sample		
		CT	CA	AR
Tyr	84–120	104.3 ± 7.7 ^b	138.7 ± 9.5 ^a	94.0 ± 12.8 ^b
	40–70	143.7 ± 5.1 ^b	170.7 ± 8.1 ^a	130.8 ± 11.6 ^b
	≤30	92.7 ± 1.8 ^b	110.6 ± 5.8 ^a	85.3 ± 4.6 ^b
Ser	84–120	99.0 ± 4.5 ^b	115.1 ± 3.5 ^a	100.1 ± 4.5 ^b
	40–45	108.3 ± 2.4 ^b	122.1 ± 4.1 ^a	107.9 ± 2.9 ^b
Thr	84–120	107.4 ± 3.2 ^b	122.7 ± 2.0 ^a	96.9 ± 7.1 ^b
	40–70	122.9 ± 12.1 ^b	157.7 ± 2.7 ^a	114.7 ± 6.9 ^c
	≤30	89.1 ± 7.9 ^b	117.6 ± 6.2 ^a	82.6 ± 10.6 ^b

Results indicate the phosphorylation signal (area × intensity) of different molecular weight regions relative to the total phosphorylation signal (100%) obtained in every case. Mean values ± s.e.m. (*n* = 3). Different superscripts in rows indicate significant differences. ^{a–b}*P* < 0.05; ^{a–c}*P* < 0.01.

Figure 3 Indirect immunofluorescence localization of phosphotyrosine proteins in control (0 h, A–B), capacitated (4 h, C–D) and acrosome-reacted (E–F) ram spermatozoa using monoclonal anti-phosphotyrosine antibody and FITC-conjugated chicken anti-mouse IgG. Panels A, C and E correspond to fluorescence images. Panels B, D and F correspond to their respective bright field. Panels G, H and I show representative confocal microscopy images of control, capacitated and acrosome-reacted samples respectively. The arrows indicate different domains with specific phosphorylation: EQ, equatorial region; Ac, acrosome; P, post-acrosome; MP, middle piece; and Fl, flagellum. Magnification 1000 $\times$.

specificity of each antibody as the binding was eliminated in the presence of the competing agents. Different concentrations of the three phosphoamino acids were assayed, and the obtained results are shown in Fig. 2 (lanes 4, 5).

To prove that these protein bands are phosphorylated, sperm protein samples were treated with alkaline phosphatase for removing the phosphate groups. As shown in Fig. 2 (lane 6), most proteins were fully dephosphorylated as reactivity disappeared, and only two bands were barely visible by incubating with the primary mouse monoclonal anti-phosphotyrosine, phosphoserine, or phosphothreonine antibody.

Immunolocalization of phosphorylated proteins in ram spermatozoa during *in vitro* capacitation and acrosome reaction

The localization of tyrosine-, serine- and threonine-phosphorylated proteins and their modifications according to the functional state of ram spermatozoa were analyzed by indirect immunofluorescence and confocal microscopy, and confirmed by immunocytochemistry. Phosphoresidues were associated with specific sperm domains, and we were able to distinguish different sperm subpopulations according to their surface distribution (Figs 3, 4 and 5). The mean percentage of each subpopulation in control, capacitated and AR samples

Figure 4 Indirect immunofluorescence localization of phosphoserine proteins in control (0 h, A–B), capacitated (4 h, C–D) and acrosome-reacted (E–F) ram spermatozoa using monoclonal anti-phosphoserine antibody and FITC-conjugated chicken anti-mouse IgG. Panels A, C and E correspond to fluorescence images. Panels B, D and F correspond to their respective bright field. Panels G, H and I show representative confocal microscopy images of control, capacitated and acrosome-reacted samples respectively. The arrows indicate different domains with specific phosphorylation: EQ, equatorial region; Ac, acrosome; N, neck; An, annulus; P, post-acrosome; MP, middle piece; PP, principal piece; and FI, flagellum. Magnification 1000 $\times$.

was assessed. Less than 10% of cells were unstained for all three residues and no significant differences were found between samples. These results were highly specific because samples processed with the secondary antibody only showed no signaling (Fig. 6A and B), and replacing the antiserum with preimmune serum abolished protein detection (Fig. 6C and D). Likewise, pre-incubation of each primary antibody with phosphotyrosine, phosphoserine and phosphothreonine respectively, showed no reactivity in any case (Fig. 7). For a better analysis, the different sperm patterns of tyrosine-, serine- and threonine-phosphorylated proteins were grouped in six categories (Fig. 8).

In control samples, tyrosine phosphoproteins were located on the sperm head (Fig. 3A), predominantly at the equatorial segment (80.62%, Fig. 8A), either with or without a faint signal in the acrosomal and/or post-acrosomal region. *In vitro* capacitation promoted phosphorylation both in tail ($P < 0.001$, Fig. 8A), with differences in length and intensity from the anterior midpiece and principal piece to the whole flagellum, and punctate staining at the anterior portion of the acrosome (Fig. 3C). Finally, in A23187-stimulated samples, changes in phosphotyrosine protein localization towards the post-acrosomal and midpiece regions were found (Figs 3E and 8A). Therefore,

Figure 5 Indirect immunofluorescence localization of phosphothreonine proteins in control (0 h, A–B), capacitated (4 h, C–D) and acrosome-reacted (E–F) ram spermatozoa using monoclonal anti-phosphothreonine antibody and FITC-conjugated chicken anti-mouse IgG. Panels A, C and E correspond to fluorescence images. Panels B, D and F correspond to their respective bright field. Panels G, H and I show representative confocal microscopy images of control, capacitated and acrosome-reacted samples respectively. The arrows indicate different domains with specific phosphorylation: EQ, equatorial region; Ac, acrosome; N, neck; P, post-acrosome; MP, middle piece; and FI, flagellum. Magnification 1000 $\times$.

acrosomal exocytosis resulted in a significant loss of the acrosomal localization of phosphotyrosine proteins (69.96% in capacitated samples versus 14.38% in AR samples, $P < 0.001$). The confocal analysis confirmed these observations (Fig. 3G–I) and z-axis series images showed a subcellular localization of different tyrosine phosphoproteins (images not shown).

The distribution of proteins phosphorylated at serine and/or threonine residues was similarly analyzed. Before incubation, phosphoserine proteins were mainly distributed both in the acrosome and equatorial regions (75.53%, Fig. 8B), with or without a faint signal at the neck. This immunopattern was characterized by a bright

arch-like staining at the equatorial segment as well as a strong punctate signal forming a band in the apical ridge of the acrosome (Fig. 4A). In addition, 24.33% of cells displayed fluorescence in the tail, mainly restricted to the anterior part of the midpiece and principal piece. Capacitation promoted serine phosphorylation labeling in the flagellum ($P < 0.01$, Fig. 4C) simultaneously with a decrease in the intensity and percentage of cells with acrosomal localization (85.56% in control versus 44.19% in capacitated samples, $P < 0.001$). Tail fluorescence ranged from a partial signal located at the neck, annulus and principal piece to a strong staining in the entire flagellum. It is noteworthy that phosphorylation

Figure 6 Negative controls of indirect immunofluorescence (A–D) and immunocytochemistry (E, F). Samples incubated with only the secondary antibody (panels A, B and E). Samples incubated with preimmune serum instead of the primary antibody (panels C, D and F). Paired epifluorescent (A, C) and bright-field (B, D) pictures respectively are shown. Magnification 1000 $\times$.

in the whole flagellum was often associated with the loss of the acrosomal and equatorial distributions with 44.53% of sperm displaying labeling only in the tail ($P < 0.001$). After inducing the acrosome reaction, only 17.8% of cells showed a certain signal in the acrosome, and phosphoserine protein signaling increased in the post-acrosomal region, with 44.75% of cells also presenting tail reactivity (Figs 4E and 8B). These results were confirmed by confocal microscopy (Fig. 4G–I).

When anti-phosphothreonine antibody was used, control samples also showed fluorescence over the acrosomal and equatorial regions (80.62%), with (30.58%) or without (50.04%) punctate labeling at the connection piece (Figs 5A and 8C). A total of 80.62% of sperm were stained only on the head, while just 19.3% showed certain reactivity in the tail. Once again, the incubation in capacitation conditions promoted threonine phosphorylation in the flagellum with 51.9% of cells exhibiting only a signal in the whole tail ($P < 0.001$), and a simultaneous decrease in the acrosomal and equatorial staining ($P < 0.01$; Figs 5C and 8C). The induction of the acrosome reaction with calcium ionophore also resulted in a loss of the acrosomal labeling ($P < 0.001$), with only 14.38% of sperm maintaining this localization (Fig. 8C). Most sperm showed phosphothreonine residues distributed over the

Figure 7 Negative controls using monoclonal anti-phosphotyrosine (A, B), anti-phosphoserine (C, D), or anti-phosphothreonine (E, F) antibody pre-absorbed with phosphotyrosine (12.5 mg/ml), phosphoserine and phosphothreonine (8.5 mg/ml) respectively. Paired epifluorescent (A, C, E) and bright-field (B, D, F) pictures respectively are shown. Magnification 1000 $\times$.

post-acrosome, with (40.98%) or without signal at the flagellum, mainly the midpiece (Fig. 5E). These changes in phosphothreonine protein staining were confirmed by confocal microscopy (Fig. 5G–I).

The localization of sperm proteins phosphorylated at tyrosine, serine and threonine residues and their modifications due to *in vitro* capacitation and the acrosome reaction revealed by the avidin–biotin complex technique corroborated the previous findings (Fig. 9). These results were also highly specific because the incubation with only the secondary antibody (Fig. 6E) and replacing the antiserum with preimmune serum (Fig. 6F) abolished protein signaling.

Discussion

The involvement of the cAMP–PKA pathway in sperm capacitation (Visconti *et al.* 1997, Visconti & Kopf 1998) and acrosome reaction (Breitbart & Naor 1999, Garde & Roldan 2000) has been reported as well as the modulation of both processes by intracellular signals associated to protein phosphorylation (Aitken *et al.* 1995, Burks *et al.* 1995, Baldi *et al.* 1996, Visconti & Kopf 1998, Visconti *et al.* 2002, Naz & Rajesh 2004, Liguori *et al.* 2005, O'Flaherty *et al.* 2006a). In addition, other Ser/Thr kinases such as MAPK also appear to play a role in capacitation (de Lamirande & Gagnon 2002, O'Flaherty *et al.* 2006a, 2006b) and acrosome reaction

Figure 8 Percentage of ram spermatozoa showing different immuno-localization patterns of phosphotyrosine (A), phosphoserine (B) and phosphothreonine (C) proteins analyzed by indirect-immunofluorescence. Eq, equatorial region; Ac, acrosome; Pa, post-acrosome; N, neck; and FI, flagellum. Black bars, control; white bars, capacitated; gray bars, acrosome-reacted samples. Mean values $\pm$ S.E.M. ($n=3$). Significant differences related to control samples: * $P<0.05$; ** $P<0.01$; *** $P<0.001$. Significant differences related to capacitated samples: ## $P<0.01$, ### $P<0.001$.

(Luconi *et al.* 1998, du Plessis *et al.* 2001, de Lamirande & Gagnon 2002, Liguori *et al.* 2005), suggesting a complex regulation of these processes through different intracellular pathways (Liguori *et al.* 2005, O'Flaherty *et al.* 2006a, 2006b).

In previous studies (Perez-Pe *et al.* 2002, Grasa *et al.* 2006), we proved that protein tyrosine phosphorylation is associated to ram sperm capacitation, and that the cAMP–PKA pathway is at least partially implicated in this process. In this study, we found a certain degree of protein serine and threonine phosphorylation before incubating sperm in capacitating conditions (control samples, Fig. 2B and C). As the proportion of capacitated sperm in control samples was not very high (Fig. 1), this might have occurred either because phosphorylations started during the swim-up procedure, or because some

Figure 9 Immunocytochemical localization of phosphoproteins in control (CT), capacitated (CA) and acrosome-reacted (AR) ram spermatozoa using monoclonal anti-phospho tyrosine (A), serine (B) and threonine (C) antibody. The arrows indicate different domains with specific phosphorylation. Magnification 1000 $\times$.

proteins are constitutively phosphorylated in serine and/or threonine residues, as already reported for tyrosine residues (Grasa *et al.* 2006). After incubating in capacitating conditions, we detected a quantitative increase in the intensity of several bands, which indicates that ram sperm capacitation is also associated with protein Ser/Thr phosphorylation (Fig. 2) as occurs in other species such as humans (Naz 1999, de Lamirande & Gagnon 2002, O'Flaherty *et al.* 2004), hamsters (Jha & Shivaji 2002b), boars (Harayama 2003, Harrison 2004) and mice (Jha *et al.* 2006). All controls carried out in this study proved signal specificity. The fact that after incubation with alkaline phosphatase, certain bands were still barely visible could well be because these bands correspond not to a monoester phosphate but a phosphodiester linkage on which alkaline phosphatase exhibits no activity, as already shown (Dennis *et al.* 2000).

It is noteworthy that the increase in serine-phosphorylated proteins (mainly 84–120 and 40–45 kDa molecular mass regions) partially coincides with the increase in threonine-phosphorylated proteins (84–120 and 40–70 kDa regions; Fig. 2B and C). In addition, the increase in phosphotyrosine protein signaling (40–120 and <30 kDa molecular mass) was linked to increased phosphothreonine protein labeling (the same molecular weight regions; Fig. 2A and C). These observations would suggest that ram spermatozoa might possess dual

specificity kinases that would phosphorylate in more than one residue, as already described in other mammalian species such as humans (Naz 1999, Thundathil *et al.* 2002, Ficarro *et al.* 2003), hamsters (Jha & Shivaji 2002b) and boars (Harayama 2003, Harrison 2004).

Acrosomal exocytosis induced by calcium ionophore A23187 has been used in ram sperm to investigate the signaling pathways involved in this process (Roldan & Fragio 1993, Garde & Roldan 2000). In our study, the induction of the acrosome reaction by this compound resulted in a significant decrease in the phosphorylation signal, mainly of proteins whose phosphorylation increased during capacitation (Fig. 2). The fact that about 40% sperm maintained unreacted acrosomes might be explained by the high sperm heterogeneity that accounts for cells with a wide range of membrane properties and susceptibility. The decrease in the total phosphotyrosine content after inducing the acrosome reaction is consistent with previous observations in boar sperm (Kalab *et al.* 1998, Green & Watson 2001), and indicates that dephosphorylation may be associated with Ca^{2+} influx during the acrosome reaction (Kalab *et al.* 1998, Baker *et al.* 2004). However, it may also be due to a loss of some phosphoproteins due to the high number of sperm that have reacted acrosomes.

Differences in the localization of phosphorylated proteins in sperm have been associated with different functions (Urner & Sakkas 2003), and a specific sequence in the cellular immunolocalization of tyrosine phosphoproteins during capacitation and zona pellucida binding has been found in mice (Urner *et al.* 2001) and humans (Sakkas *et al.* 2003, Liu *et al.* 2006). Our results showed that, in control ram spermatozoa, tyrosine-phosphorylated proteins are restricted to the head, distributed mainly at the equatorial region with some cells also displaying an acrosomal and/or post-acrosomal localization, and that *in vitro* capacitation promoted both tail and acrosome phosphorylation. Similarly, several studies have reported a preferential distribution of tyrosine phosphoproteins in the flagellum of capacitated sperm such as human (Carrera *et al.* 1996, Leclerc *et al.* 1997, Sakkas *et al.* 2003), monkey (Mahony & Gwathmey 1999), mouse (Urner *et al.* 2001), hamster (Jha & Shivaji 2002a) and stallion (Pommer *et al.* 2003). The situation of phosphorylated proteins at the principal piece of the flagellum has been related to motility hyperactivation and to the presence of A-kinase anchoring proteins (which anchor PKA) located in the fibrous sheath of the sperm flagellum in several species (Carrera *et al.* 1996, Mandal *et al.* 1999, Jha & Shivaji 2002a, Pommer *et al.* 2003, Sakkas *et al.* 2003). Conversely, a capacitation-related redistribution of phosphotyrosine residues to the acrosome has been reported in humans (Naz *et al.* 1991), boars (Tardif *et al.* 2001, Dube *et al.* 2005), bulls (Cormier & Bailey 2003) and buffalo (Roy & Atreja 2008). Moreover, other studies

have revealed that certain tyrosine phosphoproteins become localized to the sperm surface overlying the acrosome during capacitation suggesting a key role of these proteins in sperm–zona binding (Asquith *et al.* 2004, Piehler *et al.* 2006). Therefore, the distribution of phosphotyrosine residues and their modifications associated to ram sperm capacitation presented in this study might indicate a role of these proteins during capacitation and in acrosome reaction regulation.

In contrast to tyrosine-phosphorylated proteins, the studies carried out to date on serine and threonine phosphorylation are relatively scarce. The preferential localization over the sperm head (Figs 4 and 5) found in control samples changed during capacitation, which induced tail phosphorylation in a sequential manner. The phosphorylation pattern ranged from sperm showing reactivity at the neck and principal piece to sperm displaying phosphoproteins all along the flagellum, as previously reported for cAMP-dependent serine/threonine phosphorylation in boar sperm (Harayama 2003). Simultaneously, a decrease in the acrosomal content of serine/threonine phosphoproteins was achieved. It is noteworthy that the bright signal of serine and threonine residues on the acrosome became punctate after capacitation, which indicates a lower content of these proteins at this region. Similarly, a preferential localization of serine/threonine-phosphorylated proteins over the tail regions has been reported in capacitated sperm of humans (Naz 1999, O'Flaherty *et al.* 2004), hamsters (Jha & Shivaji 2002b) and mice (Jha *et al.* 2006). Moreover, serine/threonine phosphorylation appears to occur early during capacitation, as reported in boar for PKA-catalyzed phosphorylation (Harayama 2003, Harrison 2004) or in mouse proline-directed serine/threonine phosphorylation (Jha *et al.* 2006), which would indicate that the activation of serine/threonine kinases is upstream of tyrosine phosphorylation.

Our data also showed that the induction of the acrosome reaction promoted the loss of phosphoprotein labeling on the acrosomal domain and the subsequent increase in the post-acrosomal region and flagellum (middle piece), which might suggest a key role for these proteins in further stages of sperm–oocyte interaction. The loss of the acrosomal localization could be related to masking, redistribution, dephosphorylation, or the loss of proteins involved in fusion membrane events as the number of sperm with reacted acrosomes is very high. Furthermore, the localization of phosphorylated proteins in the acrosome of capacitated sperm, together with its further loss after the acrosome reaction, could be related to the activation of certain proteins involved in this process (Dube *et al.* 2005). Similarly, as reported in several mammalian species, zona pellucida binding induced phosphorylation in the midpiece (Leyton & Saling 1989, Flesch *et al.* 2001) and other tail regions (Urner *et al.* 2001, Sakkas *et al.* 2003).

The results of immunofluorescence analysis were confirmed by immunocytochemistry and confocal microscopy, and allow us to postulate a subcellular localization of phosphorylated proteins in different cell compartments. Our data are consistent with those in a previous report in which we identified the presence of a seminal plasma protein in the ram sperm membrane (RSVP14, [Fernandez-Juan et al. 2006](#)), phosphorylated at serine and threonine residues, which undergoes a partial loss and redistribution during capacitation and acrosome reaction towards the post-acrosomal and equatorial regions ([Barrios et al. 2005](#)). The results of the present study support our previous suggestion that serine/threonine phosphorylation may play an important role in sperm capacitation, since consolidation of phosphoproteins to specific domains of the head and tail may facilitate the acrosome reaction and egg penetration ([Naz 1999](#)). Moreover, the similar localization and the changes described for tyrosine-, serine- and threonine-phosphorylated proteins indicates the activation of signal transduction pathways in specific sperm domains associated to specific functions, as already reported in boar sperm ([Harayama 2003](#)).

In conclusion, *in vitro* ram sperm capacitation and the acrosome reaction promoted changes in the content and subcellular localization of proteins phosphorylated at tyrosine, serine and threonine residues towards new domains involved in later stages of fertilization. These changes may have a physiological significance in consolidating certain phosphorylated proteins to specific sperm regions involved in acrosomal exocytosis and zona pellucida recognition, binding and penetration.

Materials and Methods

Sperm preparation

All experiments were performed using fresh ram spermatozoa. Semen was collected from eight mature Rasa Aragonesa rams using an artificial vagina. The rams, ranging from 2 to 4 years of age, were supplied by the National Association of Rasa Aragonesa Breeding (ANGRA), and were kept at the Faculty of Veterinary Medicine under uniform nutritional conditions.

The sires were kept apart and semen was collected every two days, in two successive matings each day (0830–0930 h). Under these conditions, and using second ejaculates, individual differences are very low, as we have already reported ([Ollero et al. 1996](#)), and pooled ejaculates provide a uniform, good quality sperm sample suitable for representative studies of ram semen.

A seminal plasma-free sperm population was obtained by a dextran/swim-up procedure ([García-López et al. 1996](#)) performed in a media devoid of NaHCO_3 and CaCl_2 , ([Grasa et al. 2004, 2006](#)). Control samples (not capacitated, NC) were taken immediately following swim-up procedure to process by immunoblotting and immunofluorescence.

In vitro capacitation and acrosome reaction

In vitro capacitation was performed by incubating the swim-up obtained samples (2×10^8 cells/ml) in the presence of 25 mM NaHCO_3 , 3 mM CaCl_2 and 5 mg/ml of BSA for 4 h at 39 °C in a humidified incubator with 5% CO_2 in air, obtaining capacitated samples (C).

Acrosome reaction was induced by the addition of 3 μM calcium ionophore A23187 (Sigma Chemical Co.) in 0.3% DMSO, Sigma Chemical Co.) to previously capacitated sperm (2×10^7 cells/ml) and further incubation at 39 °C for 1 h (AR samples, [Marti et al. 2000](#)). Control tubes had DMSO added but no ionophore, which was found to be without effect (data not shown).

Evaluation of sperm samples

Sperm concentration was calculated in duplicate using a Neubauer's chamber (Marienfeld, Germany).

Capacitation status was evaluated using the CTC assay in combination with EthD-1 staining, Molecular Probes Inc., Eugene, OR, USA), as previously described ([Grasa et al. 2006](#)). Three sperm populations, NC, capacitated (C) and AR cells, were estimated under epifluorescence illumination using a V-2A filter. For treatment of results, only the live cells were taken into consideration.

Extraction of proteins

Aliquots of 0.5 ml (10^7 cells) of NC, C and AR samples were centrifuged in a microfuge at 7500 *g* for 5 min at room temperature, and the supernatant was discarded. The resulting sperm pellet was resuspended in 100 ml extraction medium (2% SDS, 28% sucrose, 12.4 mM N,N,N,N' -tetramethylethylenediamine and 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. Finally, extracts were incubated at 100 °C for 5 min and then stored at –20 °C until required. Prior to the addition of 2-mercaptoethanol, the concentration of extracted proteins was measured using the Micro BCA Protein Assay Reagent kit (Pierce, Rockford, IL, USA).

SDS-PAGE and western blot analysis

Solubilized proteins were separated by electrophoresis on 4–22.5% (w/v) SDS-PAGE gels ([Laemmli 1970](#)) and electrotransferred onto Immobilon-P (Millipore, Bedford, MA, USA) as previously described ([Perez-Pe et al. 2002](#)). Non-specific binding sites on membranes were blocked with 5% BSA (w/v) in Tris-buffered saline (10 mM Tris-HCl, pH 8, 120 mM NaCl and 0.05% Tween 20) for 1 h. The phosphoproteins were immunodetected by incubating with the primary mouse monoclonal anti-phosphotyrosine (1:2000), phosphoserine (1:4000), or phosphothreonine (1:4000) antibody (PT-66, PSR-45 and PTR-8 respectively, Sigma Chemical Co.) for 3 h

at room temperature. After several washes in Tris-buffered saline, the membranes were incubated for 1 h with a secondary goat anti-mouse alkaline phosphatase-conjugated IgG (1:4000; Sigma Chemical Co.). Thereafter, phosphorylated proteins were visualized by incubation with a substrate mixture of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (0.33 mg/ml in dimethyl formamide) in 0.2 M Tris, 5 mM MgCl₂ and NaCl 0.1 M, pH 9.6. Finally, the membranes were washed with distilled water, dried, and scanned.

Western blot images were analyzed using Scion Image software (Scion Co., Frederick, Ma, USA) to quantified changes in the phosphorylated proteins. The phosphorylation signal was evaluated as volume (area $\times$ intensity), and results are shown as a percentage relative to the total phosphorylation signal obtained in each case.

The specificity of each antibody was proved by incubating each primary antibody with the competing agent phosphotyrosine, phosphoserine and phosphothreonine respectively, overnight at 4 °C. Different concentrations of the three phosphoamino acids (25, 50, 100, 500 and 1000 µg/ml) were assayed.

In addition, to prove that these proteins are phosphorylated, sperm protein samples (100 µg) were incubated with 23 U alkaline phosphatase, 14 mM NaCl, 0.5 mM MgCl₂, 0.5 mM ZnCl₂ and 140 µM triethanolamine, overnight at 37 °C.

Indirect immunofluorescence

The localization of proteins phosphorylated at tyrosine, serine and threonine residues of NC, C and AR samples was investigated by indirect immunofluorescence. Sperm samples were washed with PBS (pH 7.2) and fixed for 30 min in 4% of paraformaldehyde. After fixation and washing, cell samples were allowed to settle on poly-L-lysine-coated slides and permeabilized by methanol for 15 min. The slides were then washed twice with PBS, and non-specific binding sites were blocked with 5% BSA in PBS for 1 h at 37 °C in a humid chamber. After blocking, the slides were washed three times with PBS and anti-phosphotyrosine (PT-66), anti-phosphoserine (PSR-45), or anti-phosphothreonine (PTR-8) mouse antibodies (Sigma Chemical Co.) 1:200, 1:400, 1:400 in PBS with 0.5% BSA respectively were added. The preparations were incubated for 2 h at 37 °C in the wet chamber, washed three times and incubated again with a fluorescein isothiocyanate (FITC)-conjugated chicken anti-mouse IgG (Molecular Probes, Leiden, The Netherlands; 1:500 diluted in PBS with 0.5% BSA) for 1.5 h at 37 °C in darkness. The slides were rinsed with PBS and mixed with 5 µl 0.22 M triethylenediamine (DABCO, Sigma Chemical Co.) in glycerol:PBS (9:1) in order to enhance and preserve cell fluorescence. Finally, the preparations were covered with cover slips, sealed with colorless enamel, and visualized under a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) under epifluorescence illumination using a B-2A filter. At least 200 cells per sample were evaluated and the percentage of cells displaying positive fluorescence was scored. Negative controls were performed by processing slides with either the secondary antibody alone, or with mouse preimmune serum (diluted with PBS 1:100) instead

of the primary antibody. In addition, another control using each primary antibody (anti-phosphotyrosine 1:200, anti-phosphoserine 1:400, or anti-phosphothreonine 1:400 in PBS with 0.5% BSA) pre-incubated with phosphotyrosine (12.5 mg/ml) phosphoserine (8.5 mg/ml) and phosphothreonine (8.5 mg/ml), overnight at 4 °C, was carried out. Images were obtained using a microscope digital camera system (Sony 2CCD Color Video Camera and Sony Digital Still Recorder) and saved and edited with Visilog 5.1 Software (Microptic, Barcelona, Spain).

The localization patterns of phosphoproteins observed by immunofluorescence were confirmed by three-dimensional confocal microscopy analysis, performed with a confocal microscope (Zeiss 310) using LSM 3.95 software. Individual cells were observed within 10 successive focal planes separated 0.1–0.2 µm.

Immunocytochemical technique

The spermatozoa were exposed to immunocytochemical staining by using an avidin–biotin–peroxidase technique (Vector, Los Angeles, CA, USA). Sperm samples were smeared onto poly-L-lysine-coated slides and fixed in methanol as described above. After rehydration in PBS pH 7.2, endogenous peroxidase was inactivated with 1.7% hydrogen peroxide in 100% ethanol for 30 min. Subsequently, they were washed with PBS pH 7.2, and then incubated in normal horse serum (blocking reagent; Vector) for 45 min, followed by incubation for 2 h 30 min with the specific antiserum (primary antibody) as described for indirect immunofluorescence. After washing, the slides were incubated with biotinylated anti-mouse antiserum for 40 min. An avidin–biotin–peroxidase complex was then applied for 45 min. The binding sites of the primary antibodies were visualized by diaminobenzidine (DAB) and hydrogen peroxide solution (20 mg DAB in 100 ml of 0.05 M Tris–HCl buffer, pH 7.6, containing 0.005% H₂O₂) for 5 min. As negative controls, samples were incubated with preimmune serum instead of the primary antibody, with the remaining procedure being the same. In these conditions, no reactivity was found. Likewise, slides were processed with the secondary antibody alone as another negative control. The images obtained were taken using the same microscope digital camera system and software as for immunofluorescence.

Statistical analysis

Results are shown as mean $\pm$ S.E.M. of the number of samples indicated in each case. ANOVA was performed to determine whether there were significant differences between samples, and post hoc comparisons were made using Tukey's test. Software used was GraphPad InStat (San Diego, CA, USA).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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