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Capacitation-associated Changes in Protein-tyrosine-phosphorylation, Hyperactivation and Acrosome Reaction in Guinea Pig Sperm*

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ABSTRACT : The aim of this study was to evaluate the effects of Ca^{2+}, HCO_3^- and BSA on the *in vitro* capacitation-associated protein tyrosine phosphorylation, hyperactivation and acrosome reaction in guinea pig sperm. Caudal epididymal sperm were incubated in four different groups: modified TALP (Tyrode's albumin lactate pyruvate) or TALP without one of the medium constituents (Ca^{2+}, HCO_3^- and BSA). After incubation for the required time (0 h, 0.5 h, 1 h, 3 h, 5 h, and 7 h), sperm were removed for further experiment. The capacitation effect was assessed by CTC (Chlortetracycline) staining. Western blotting and indirect immunofluorescence were used to analyze the level and localization of tyrosine phosphorylation. The results showed that guinea pig sperm underwent a time-dependent increase in protein tyrosine phosphorylation during the *in vitro* capacitation and the percentage of protein tyrosine phosphorylated sperm increased from 36% to 92% from the beginning of incubation to 7 h incubation. Also, there was a shift in the site of phosphotyrosine-specific fluorescence from the head of sperm to both the head and the flagellum. Moreover, an absence of Ca^{2+} or HCO_3^- inhibited *in vitro* hyperactivation and acrosome reaction and decreased the phosphorylation of the proteins throughout the period of *in vitro* capacitation. However, an absence of BSA could not influence these processes if substituted by polyvinyl alcohol (PVA) in the medium.
(**Key Words:** Acrosome Reaction, Capacitation, Hyperactivation, Tyrosine Phosphorylation, Semen)

INTRODUCTION

After leaving the testis, mammalian sperm are morphologically differentiated but are immotile and unable to fertilize. Progressive motility is acquired during epididymal transit and fertilization capacity is gained when sperm passed through the female reproductive tract in a process called capacitation (Harayama and Kato, 2001; Marquez and Suarez, 2004; Naz and Rajesh, 2004). Capacitation involves several changes, occurring in both the sperm head and tail that lead to the release of the acrosomal content (called acrosome reaction, AR) (Yanagimachi, 1994) and to the acquisition of a distinct type of motility known as hyperactivation (Si and Okuno, 1999). Both events are essential for sperm penetration through the egg coatings.

However, little is known about the molecular basis of sperm capacitation. Since ejaculated or caudal epididymal mammalian sperm can be capacitated *in vitro* by using a defined medium, the *in vitro* capacitation system has been used to understand the molecular basis of capacitation. It has been shown that certain components in the medium promote capacitation, such as Ca^{2+}, HCO_3^- and serum albumin (as the primary protein source). For instance, the reports on mouse (Visconti et al., 1995) and human sperm (Lecerc et al., 1998) have documented that increasing amounts of extracellular Ca^{2+} increase tyrosine phosphorylation. In contrast, other studies have demonstrated the opposite effect (Carrera et al., 1996; Luconi et al., 1996) indicating that Ca^{2+} negatively regulates tyrosine phosphorylation during *in vitro* capacitation. The HCO_3^- influx has been associated with an increase in intracellular pH observed during the capacitation (Zeng et al., 1996), regulation of cAMP levels, reversible change in the lipid architecture of plasma membrane, and hyperpolarization of sperm plasma membrane (Boatman and Robbins, 1991). Serum albumin, usually BSA, presented in the capacitation media for mammalian sperm (e.g., mouse, hamster, cattle, and human), is believed to function during *in vitro* capacitation as a sink for the removal of cholesterol from the sperm plasma membrane

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(Langlais and Roberts, 1985; Huang et al., 2000). But in the capacitation process of guinea pig sperm, it's still not known the effect of Ca^{2+}, HCO_3^- and BSA in the *in vitro* medium and which process they would influence in the complex series of molecular events.

In this study, we used conditions that are conductive to sperm *in vitro* capacitation in guinea pig and attempted to investigate the following: i) How Ca^{2+}, $NaHCO_3$ and BSA effect hyperactivation and acrosome reaction? ii) How protein tyrosine phosphorylation changes during capacitation? iii) How Ca^{2+}, $NaHCO_3$ and BSA influence protein tyrosine phosphorylation?

MATERIALS AND METHODS

Animals

Male guinea pigs (Nanjing Qingzilan Technology CO., Ltd., China), weighted 720-750 g, were used in the experiments. The investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals.

Sperm preparation

Mature guinea pigs were killed by ethyl ether inhalation. Caudal epididymides were excised. After removal of blood from the epididymal surface with tissue paper, the distal portion of the epididymis was pierced with a syringe needle, and the exudates were released directly into TALP (Tyrode's albumin lactate pyruvate) (They were stored at 37°C in a CO_2 incubator, which was continuously flushed with 5% CO_2), a modified Tyrode's medium demonstrated to support *in vitro* capacitation (Jha and Shivaji, 2002). TALP was prepared in deionised water as an inorganic salt solution containing sodium chloride (114 mM), potassium chloride (3.16 mM), magnesium chloride hexahydrate flakes (0.35 mM), sodium hydrogen carbonate (25.07 mM), glucose (5 mM), sodium pyruvate (0.25 mM), sodium lactate (12.5 mM), benzylpenicillin potassium (10,000 IU), calcium chloride (2.0 mM). Just prior to use, BSA (3 mg/ml) was added, and the medium was allowed to equilibrate overnight at 37°C in the CO_2 incubator. TALP medium used in the present study was always supplemented with polyvinyl alcohol (PVA, 1 mg/ml of medium). In order to evaluate the influence of Ca^{2+}, $NaHCO_3$ and BSA on protein tyrosine phosphorylation, sperm suspensions were prepared in TALP or TALP without one of the constituents (Ca^{2+}, $NaHCO_3$ or BSA) and also in TALP without both BSA and PVA. When $NaHCO_3$ was excluded in TALP medium, it was supplemented with 25 mM HEPES to maintain the pH. When the mediums of all the treatments above at different pHs were used, the pH was adjusted to 7.8 with 1 mM NaOH.

Assessment of capacitation

The CTC (Chlortetracycline) method (Pietrobon et al., 2001) was used to assess the state of capacitation. Chlortetracycline is the classical stain used to detect the sperm acrosome reaction and capacitation. This probe binds to the surface of sperm and discriminates sperm noncapacitated, capacitated and acrosome reacted by different staining patterns. In briefly, the CTC solution was prepared by dissolving CTC-HCl at a concentration of 500 μM in a buffer containing 20 mM Tris.HCl, 130 mM NaCl, and 5 mM cysteine, pH 7.8. Fresh CTC solution was prepared before each assay. 50 μl of sperm suspension from each condition was mixed with 50 μl of CTC. After 20 seconds, sperms were fixed by the addition of 8 μl of 12.5% glutaraldehyde in PBS (20 mM phosphate buffer, 150 mM NaCl pH 7.4). 20 μl sperm suspension was placed on a clear slide. Sperm were examined for CTC fluorescence at a magnification of 400 on a Nikon microscope. A total of 100 sperm were scored on each slide. Three CTC staining patterns were observed: 1) capacitation was characterized by head fluorescence with a fluorescence-free band in the post acrosomal region; 2) sperm either with a weakly homogeneous fluorescence or absence of fluorescence was characteristic of acrosome-reacted cells; 3) the whole sperm with homogeneous fluorescence was referred as noncapacitation.

Assessment of hyperactivation

Hyperactivation is a type of sperm motility. Hyperactivated sperm motility is characterised by high amplitude, asymmetrical beating pattern of the sperm tail. Sperm can hyperactive during *in vitro* capacitation. Hyperactivation was assessed in motile guinea pig sperm according to the procedure described by Zhang (Zhang et al., 2000). Briefly, non-hyperactivated guinea pig sperm showed a planar motility pattern, unlike hyperactivated sperm which showed helical and circular motility patterns. By looking at motility pattern, we could, therefore, distinguish between non-hyperactivated and the hyperactivated sperm. Sperm were assessed by phase-contrast microscopy (×400). For each treatment, four or five random fields were analyzed and the percentage of motile sperms that were hyperactive was obtained, 100 sperm were scored for each point.

SDS-PAGE and western blotting

Proteins from sperm were analyzed by SDS-PAGE and Western blottingm after incubation for the required time (0 h, 0.5 h, 1 h, 3 h, 5 h, and 7 h). Cells were washed twice with phosphate-buffered saline (PBS) and re-suspended in Laemmli sample buffer (25 mM Tris, 0.5% SDS and 5% glycerol, pH 6.8) (Luconi et al., 2005). Samples were centrifuged at 6,000 g for 5 min. The supernatants were

recovered and heated at 100°C for 5 min in the presence of 70 mM 2-mercaptoethanol and stored at -20°C until use. Solublized proteins obtained from 2×10^6 sperm per lane were separated on 12% polyacrylamide gels under denaturing conditions. Pre-stained molecular weight markers (Fermentas, #SM441) were run in parallel. For Western blot analysis, proteins were electroblotted and transferred onto PVDF (Sunshine, Cat#:66543-1) at 70 V at 4°C for 2.5 h. To block non-specific binding sites, the membrane was first blocked with 2% dry skimmed milk in PBS-T (PBS containing 0.1% Tween 20). Then it was incubated for 1 h with the monoclonal anti-phosphotyrosine antibody PY100 (Cell Signaling, #9411), diluted 1: 2,000 in blocking solution (PBS containing 0.1% Tween 20). After four washes with PBS-T, an anti-mouse peroxidase-conjugated IgG diluted 1:1,000 in blocking solution was added. Following 1 h of incubation, the membrane was washed four times with PBS-T, and reactive bands were detected using the ECL kit (Applygen, #P1010 and #P1020) according to the manufacturer's instructions. All incubations were performed at room temperature. Net intensity was analyzes by Kodak 1 D Image Analytical System. The specificity of the anti-phosphotyrosine antibody was checked by treating with preimmune serum. Further, secondary antibody alone served as a control.

Stripping PVDF membranes

In order to confirm equal loading of protein, blots that had been probed for tyrosine phosphorylated proteins were stripped and reprobed with an antibody against α-tubulin. For this procedure, approximately 30 ml of stripping buffer, consisting of 2% (w/v) SDS, 62.5 mM Tris, pH 6.7, 100 mM 2-mercaptoethanol, was added to the membrane for 1 h with constant shaking at 65°C. The membrane was then washed (3×10 minutes in PBS-T), blocked and probed with the primary antibody as described by Baker (Baker et al., 2004).

Indirect immunofluorescence

Immunofluorescence was employed to examine the subcellular localization of proteins phosphorylated in tyrosine residues. Sperms were incubated during various periods of time (0 h, 0.5 h, 1 h, 3 h, 5 h, and 7 h) and washed twice with phosphate-buffered saline (PBS). Sperm concentration was adjusted to 2×10^6 cells/ml and 15 μl of the sperm suspension was spotted onto clean glass slides. Cells were air-dried on the slides, fixed and permeabilized with methanol for 30 min at room temperature. The slides were incubated with the monoclonal anti-phosphotyrosine antibody PY100 (Cell Signaling, #9411), diluted 1:20 in PBS-0.1% BSA (PBS containing 0.1% BSA), for 1.5 h at room temperature in a humidified chamber. After washing twice with PBS, slides were incubated with goat anti-mouse IgG conjugated with rhodamine conjugated goat ani-mouse IgG (Calbiochem, #401217), diluted 1:20 in PBS-0.1% BSA for 30 min at room temperature in a humidified chamber. Following the incubation, slides were washed with PBS three times, air-dried and mounted with Vectashield (Vector Laboratories, Inc.). Sperms were examined using a fluorescence microscope. 200 cells were counted in different fields and the percentage of sperm showing fluorescence was calculated. The specificity of the anti-phosphotyrosine antibody was checked by treating with preimmune serum. Further, secondary antibody alone served as a control.

Statistical analysis

Data were present as the mean±SD of multiple experiments. Significant differences between treatments were determined by the LSD test after ANOVA.

RESULTS

Effects of Ca^{2+}, HCO_3^- and BSA on motility and hyperactivation of guinea pig sperm

In TALP the percentage of motile sperm when they were incubated up to 7 h was around 80%, the percentage of hyperactivated sperm increased from around 20% at 1 h to about 70% by 5 h, the percentage of capacitated sperm increased from 1 h to 7 h when 65% of the sperm were capacitated at 7 h (Table 1).

Absence of Ca^{2+} in TALP did not affect motility but hyperactivation was inhibited (Table 1). Removing $NaHCO_3$ from TALP caused a significant decline both in motility and in hyperactivation (Table 1). However, absence of BSA could not influence neither the motility nor the process of hyperactivation (Table 1).

Effect of BSA on the capacitation of guinea pig sperm

In order to find out whether these components of the medium are essential for sperm capacitation, guinea pig sperms were incubated in TALP devoid of BSA, and the percentage of sperms undergoing capacitation was measured. When guinea pig sperms were incubated in TALP, they showed a time-dependent increase in the percentage of capacitated sperm (Table 1). Also, it was observed that guinea pig sperm incubated in TALP devoid of BSA (but containing PVA) showed a time-dependent increase in capacitation, and the percentage of capacitated sperm was different from the control only at the beginning of incubation.

Similar experiments in TALP without Ca^{2+} or HCO_3^- were not carried out because elimination of these constituents caused a significant inhibition of hyperactivation, which made the monitoring of capacitation very difficult.

Table 1. Motility, hyperactivation and capacitation ratios of caudal epididymal guinea pig sperms capacitated in TALP, TALP minus Ca^{2+}, TALP minus HCO_3^-, TALP minus BSA

	0 h	1 h	3 h	5 h	7 h	9 h
Motility						
TALP	79.33±1.53	79±2	77.67±2.08	77.67±1.53	77±1	75.33±4.73
TALP-Ca^{2+}	77.67±4.73	74.67±3.21	76.67±5.03	78±4	76.67±3.51	70.67±5.03
TALP-HCO_3^-	18±1*	20.67±3.21*	18.33±1.53*	16.33±1.53*	13±1*	10±2.31
TALP-BSA	77.33±3.79	78.33±4.16	68.33±3.05	69.67±1.15	69±4	65.33±4.16
Percentage of hyperactivation sperms						
TALP		20.33±1.53	66.67±0.58	65.33±2.08	60.67±1.73	57.33±1.53
TALP-Ca^{2+}		3.67±2.08*	5±1.73*	5.33±1.53*	2.33±2.31*	1.67±0.58*
TALP-HCO_3^-		3.33±1.53*	2.67±2.52*	1.67±4.04*	2±2*	2±2*
TALP-BSA		18.67±2.52	65±2	60.33±2.52	60.67±2.89	55.67±2.52
Percentage of capacitation						
TALP		16.67±2.52	19.33±1.15	48.33±2.08	65.67±2.89	60±1.15
TALP-BSA		5.33±2.52*	31±2*	47.33±2.52	63.33±2.08	61.33±2

Data are expressed as mean±SD (n = 5), (*) the value was different from the control, $p<0.01$.

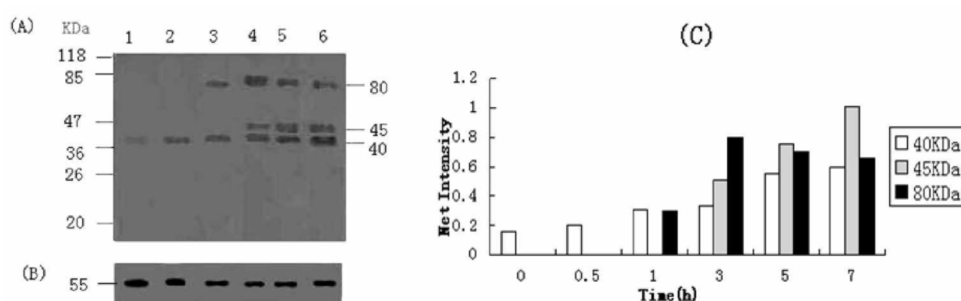

Figure 1. (A) Immunoblot analysis of tyrosine phosphorylated proteins of caudal epididymidal sperm capacitated in TALP for 0, 0.5, 1, 3, 5, 7 h (lanes 1-6, respectively). The figures on the left indicate molecular weight of the prostained marker proteins and the figures on the right indicate molecular weight of the phosphorylated proteins. (B) The PVDF membranes were stripped at 65°C with shaking. Following this the membrane was blocked and reprobed using α-tubulin antibody. The western blot is representative of 5 independent experiments. (C) The ratio of net intensity for phosphorylated proteins corroborating Figure 1.

Time-dependent changes in protein tyrosine phosphorylation of guinea pig sperm during capacitation

Western blot analysis showed that the anti-phosphotyrosine monoclonal antibody detected only one protein of 40 kDa in sperm extracts taken at 0 to 0.5 h incubation (Figure 1, lane 1-2). After 1 h incubation, the antibody recognized another protein of 80 kDa and the level of this protein reached the highest point at 3 h, when the ratio of net intensity reached 0.8 (Figure 1, lane 3-6). Furthermore, after 3 h incubation, three proteins of 40, 45, 80 kDa cross-reactive to the antibody were observed, and the intensity of 45 kDa increased since 3 h incubation (Figure 1, lane 4-6).

Localization of the tyrosine phosphorylated proteins of guinea pig sperm during capacitation

Guinea pig sperms incubated in TALP at 0 h, 0.5 h, 1 h, 3 h and 7 h were analyzed by indirect immunofluorescence, using the monoclonal anti-phosphotyrosine antibody, to localize the tyrosine phosphorylated proteins. Rhodamine conjugated goat anti-mouse IgG was used as the second antibody. Without the culture, 31% sperms were found with fluorescent signal on the head and only 5% sperm had very faint and patchy fluorescence in the tail (Figure 2a; Table 2). During the incubation, there was a shift in the site of phosphotyrosine-specific fluorescence from the head of sperm to the flagellum, and at 3 h incubation, 72% sperm had tyrosine phosphorylated proteins at the whole head and both the principal and midpiece of the flagellum (Figure 2b, c, d; Table 2). With 7 h incubation, the fluorescent signal was detected in the tail as well as in the head region and the percentage of tyrosine phosphorylated sperm was increasing to 92% (Figure 2f; Table 2).

Effects of Ca^{2+}, HCO_3^- and BSA on protein tyrosine phosphorylation of capacitated guinea pig sperm

Influences of Ca^{2+}, HCO_3^- and BSA in protein tyrosine phosphorylation during the capacitation process were studied in guinea pig sperms after incubation in TALP for 1, 3, 5 and 7 h. The absence of Ca^{2+} or HCO_3^- in TALP caused

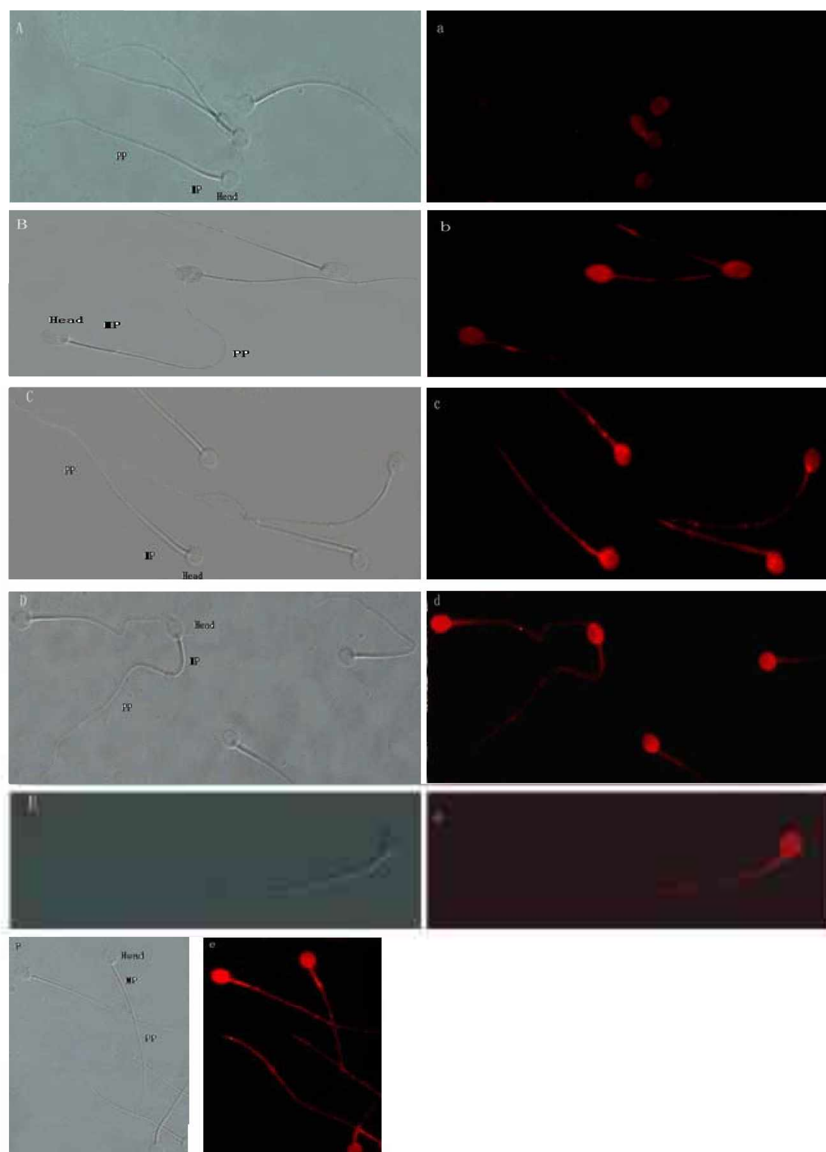

Figure 2. Indirect immunofluorescent localization of phosphotyrosine proteins on guinea pig sperm during capacitation at 0 h (A, a), 0.5 h (B, b), 1 h (C, c), 3 h (D, d), 5 h (E, e), 7 h (F, f). A-F: control photos, a-f: experimental photos (400×). MP: the middle piece of the flagellum; PP: the principal piece of the flagellum. The results are representative of 5 independent experiments.

Table 2. Precentage of tyrosine phosphorylated sperms during capacitation

	0 h	0.5 h	1 h	3 h	5 h	7 h
Sperm head	30.67±1.25	4±3.56	-	-	-	-
Sperm head and tail	5±3.27	33.33±3.86	58±2.16	72±4.55	88±4.55	92±5.10

Data are expressed as mean±SD (n = 5).

a decrease of protein tyrosine phosphorylation after up to 1 h of incubation. The data of net intensity indicated a decrease in the 40 kDa by 50% in absence of Ca^{2+} after 1 h (Figure 3A; Table 3). When guinea pig sperms incubated for 3 h, 5 h and 7 h, the 40 kDa protein was decreased by 38.7%, 46.2% and 8.8%, and the 45 kDa protein was inhibited to the extent of 50.8%, 27.3% and 16.8%, and the 80 kDa protein was inhibited to the extent of 57.1%, 50%, 18.8% and 18.8%, respectively (Figure 3A; Table 3). Similarly, when HCO_3^- were absent and guinea pig sperms were incubated for 1, 3, 5 and 7 h, the intensity of 40 kDa protein was decreased by 26.7%, 26.5%, 27.5% and 28.3%, and the 45 kDa protein was inhibited to the extend of 8.9%, 4.0% and 40.0%, the decrease in 80 kDa protein was 19.3%, 53.2%, 31.3% and 30.8%, respectively (Figure 3C; Table 3).

Guinea pig sperms incubated in TALP without BSA did not show significant change in phosphorylation of the phosphotyrosine proteins (Figure 3E; Table 3). However,

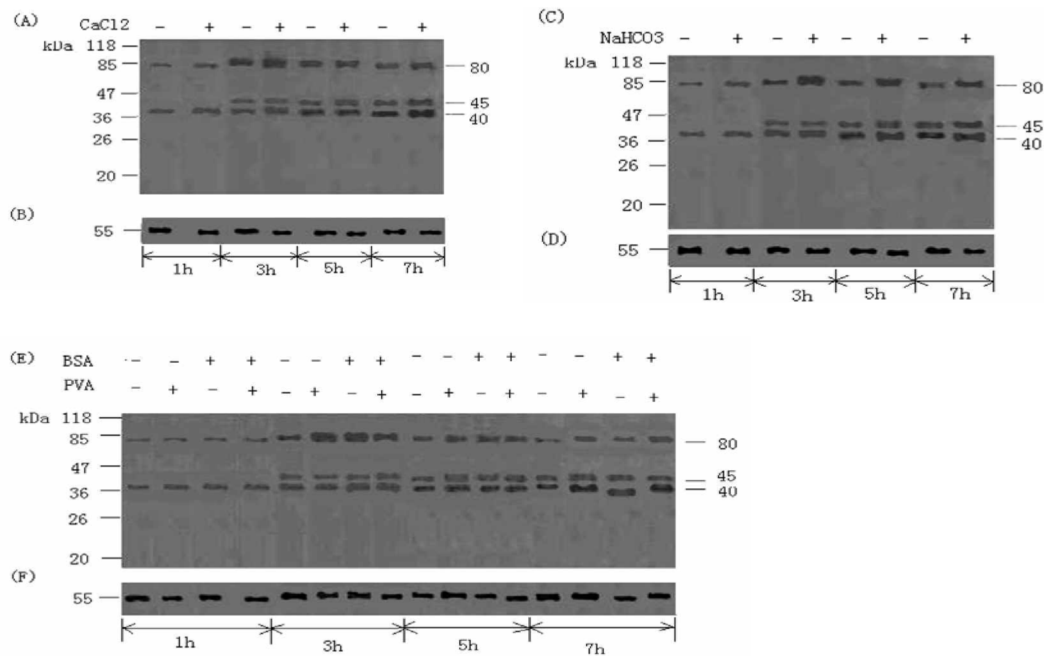

Figure 3. (A, C, E) Immunoblot analysis for the effect of Ca^{2+}/$NaHCO_3$/BSA on tyrosine phosphorylated proteins of caudal epididymidal sperm capacitated in TALP for 1, 3, 5, 7 h (lanes 1-8, respectively). "+" and "-" indicate the presence or absence of Ca^{2+}/$NaHCO_3$/BSA/PVF. The figures on the left indicate molecular weight of the prostained marker proteins and the figures on the right indicate molecular weight of the phosphorylated proteins. (B, D, F) The PVDF membranes were stripped at 65°C with shaking. Following this the membrane was blocked and reprobed using α-tubulin antibody. The western blot is representative of 3 independent experiments.

when both BSA and PVA were removed from TALP. a significant decrease in phosphorylation of the 40 kDa, 45 kDa and 80 kDa proteins was observed after up to 1 h of incubation (Figure 3E; Table 3).

DISCUSSION

Mature sperm are terminally differentiated and specialized. They are highly compartmentalized but are devoid of any major transcriptional and translational activity. Therefore, phosphorylation of proteins, which is a post-translational modification event that acts as one of the cell's regulatory mechanisms to control various processes, is important in regulation of sperm capacitation, hyperactivation and acrosome reaction. The present study demonstrated that there was a time-dependent increase in protein tyrosine phosphorylation in sperm from guinea pigs and this increase was correlated with sperm capacitation. which confirmed the recent observation of Buffone et al. (2005).

Many authors reported that four sets of tyrosine phosphorylated proteins in the molecular weight range of 95 kDa/94±3 kDa (FA-2 antigen), 46±3 kDa, 25±7 kDa and 12±2 kDa, respectively, in human sperm (Naz et al., 1991) and also identified a protein of molecular indentity of 94±3 kDa in mouse sperm. which was reported earlier by Leyton and Saling (1989). However, this protein of 94±3 kDa was not identified in rat, rabbit sperm (Naz and Rajesh, 2004) and guinea pig sperm in the present study. Although it needs to be confirmed using molecular cloning and sequencing studies. it seems that 94±3 kDa is not an envolutionarily conserved protein. The 46±3 kDa protein was found out to be the FA-1 antigen, which has been known to play an important role in capacitation (Kaplan and Naz, 1992; Naz and Zhu, 2002). but we need further experiments to identify the 40 kDa or 45 kDa protein found in the present study to see if one of them is FA-1 antigen. Tyrosine phosphorylation of 80 kDa protein appeared to be maximal at 3 h of incubation. when most of sperm were hyperactivated. Furthermore, this protein tyrosine phosphorylation could be affected when Ca^{2+}or HCO_3^-were absent in TALP. These results were similar to the observation in tyrosine phosphorylation of a kinase achoring protein 3 (AKAP3) in human sperm (Luconi et al., 2005). However, further experiments would be needed to identify this 80 kDa protein to see if it is one member of AKAPs.

In order to establish the link between the different tyrosine phosphorylated proteins and a specific sperm function. it is necessary to differentially localize the tyrosine phosphorylated proteins in various regions of guinea pig sperm. In the study. immunofluorescent staining

Table 3. The ratio of net intensity for phosphorylated proteins from sperm incubated in TALP, TALP minus Ca^{2+}, TALP minus HCO_3^- or TALP minus BSA or PVA

Molecular weight	Treatment	1 h	3 h	5 h	7 h
TALP minus Ca^{2+}					
40 kDa	TALP-Ca^{2+}	0.16±0.01*	0.19±0.004*	0.35±0.03*	0.52±0.05
	TALP	0.32±0.03	0.31±0.01	0.65±0.05	0.57±0.06
45 kDa	TALP-Ca^{2+}	-	0.31±0.07*	0.64±0.02*	0.84±0.04*
	TALP	-	0.63±0.14	0.88±0.02	1.01±0.05
80 kDa	TALP-Ca^{2+}	0.15±0.02*	0.58±0.02*	0.56±0.06*	0.52±0.04*
	TALP	0.35±0.04	1.16±0.04	0.69±0.07	0.64±0.05
TALP minus HCO_3^-					
40 kDa	TALP-HCO_3^-	0.22±0.03*	0.25±0.03*	0.37±0.07*	0.43±0.02*
	TALP	0.30±0.04	0.34±0.04	0.51±0.09	0.60±0.03
45 kDa	TALP-HCO_3^-	-	0.41±0.11*	0.72±0.05	0.70±0.04*
	TALP	-	0.45±0.12	0.75±0.06	1.06±0.07
80 kDa	TALP-HCO_3^-	0.17±0.06*	0.37±0.17*	0.44±0.03*	0.45±0.09*
	TALP	0.26±0.09	0.79±0.37	0.64±0.05	0.65±0.01
TALP minus BSA or PVA					
40 kDa	TALP-BSA-PVA	0.29±0.01*	0.18±0.02*	0.44±0.04*	0.30±0.02*
	TALP-BSA	0.37±0.04	0.43±0.03	0.65±0.05	0.63±0.02
	TALP-PVA	0.40±0.003	0.41±0.02	0.60±0.01	0.65±0.04
	TALP	0.42±0.02	0.43±0.02	0.60±0.01	0.69±0.03
45 kDa	TALP-BSA-PVA	-	0.31±0.01*	0.36±0.01*	0.41±0.01*
	TALP-BSA	-	0.54±0.03	0.53±0.03	1.06±0.03
	TALP-PVA	-	0.51±0.01	0.69±0.09	1.07±0.002
	TALP	-	0.59±0.01	0.74±0.06	1.08±0.02
80 kDa	TALP-BSA-PVA	0.20±0.01*	0.30±0.01*	0.39±0.02*	0.22±0.01*
	TALP-BSA	0.31±0.12	0.76±0.05	0.64±0.03	0.63±0.02
	TALP-PVA	0.25±0.005	0.81±0.06	0.65±0.03	0.63±0.01
	TALP	0.27±0.01	0.74±0.06	0.62±0.01	0.67±0.01

Data are expressed as mean±SD (n = 3), (*) the value was different from the control, $p<0.05$.

revealed that tyrosine phosphorylated proteins were localized both on the head and the flagellum of guinea pig sperms, which was similar with others. Except in boars (Petrunkina et al., 2001; Tardif et al., 2001), the flagellum appears to be the principal sperm compartment presenting tyrosine phosphorylated proteins. Immunocytochemistry has been used to localize tyrosine phosphorylated proteins to the flagellum in human (Naz et al., 1991; Carrera et al., 1996), monkey (Mahony and Gwathmey, 1999), hamster (Si and Okuno, 1999), rat (Lewis and Aiken, 2001), and mouse (Urner et al., 2001a) sperm.

Tyrosine phosphoryaltion in the sperm head represents a minor pattern of phosphorylation in mouse sperm. Non-capacitated sperm are all non-phosphorylated, except for about 5-10% which display protein tyrosine phosphorylation restricted to the acrosome region (Leyton and Saling, 1989). In contrast to tyrosine-phosphorylation in the flagellum, the proportion of sperm displaying a tyrosine-phosphorylated acrosome does not increase with capacitation (Urner et al., 2001). However, in rats, tyrosine phosphorylation in the acrosome region is characteristic of immature sperm from the caput epididymidis (Lewis and Aitken, 2001). This pattern of phosphorylation decreases from 100 to 10-20% with maturation in the rat epididymis and the acrosome-positive spermatozoa that persist in the cauda epididymidis have been interpreted as representative of remaining immature sperm. Also, different compartments of human sperm undergo a specific sequence of phosphorylation during capacitation and upon binding to zona pellucida (Sakkas et al., 2003). Moreover, in dog, as in guinea pig reported here, a transition of patterns has been observed during capacitation; however, the course of transition in the dog appears to be from flagellum to head, not from head to flagellum like in the present results. The reason may be that different species have different localization of tyrosine phosphorylation proteins, but the causes of these observations are still unknown.

Based on our results, it could be argued that those key components of culture media required to support capacitation also played an obligatory role in bringing about the changes in protein tyrosine phosphorylation. Guinea pig sperms incubated in TALP without Ca^{2+} or HCO_3^- showed a decrease in protein tyrosine phosphorylation and simultaneously an inhibition of hyperactivation. This indicated a possible correlation between decrease in protein tyrosine phosphorylation and inhibition in hyperactivation.

The requirement of extracellular Ca^{2+}or HCO_3^- for both protein tyrosine phosphorylation and capacitation also represented a novel regulatory mechanism of cellular signaling, since these ions have been shown to be activation of the mammalian sperm adenylyl cyclase. It has been demonstrated that this adenylyl cyclase, responsible for the synthesis of cAMP, is stimulated directly or indirectly by Ca^{2+} and HCO_3^- (Visconti and Kopf, 1998; Gadella and Gestel, 2004). Also, it is reported that capacitation is a calcium-dependent process, which is related to a myriad of biochemical changed. These changes results in outstanding increases in cAMP content and PKA activation, and in an increase in tyrosine kinase activity (Neri-Vidaurri et al., 2006). However, we were unable to confirm the results of Baker (2004) that Ca^{2+} suppresses tyrosine phophorylation by decreasing the availability of intracellular ATP. These different observations may come from the complexity of studying the effects of Ca^{2+} and their related processes.

BSA, as an extracellular sink for sperm membrane cholesterol efflux and the consequent alternation in membrane fluidity and membrane destabilization, would help to accelerate those ionic changes, which would ultimately result in the capacitated state (Visconti et al., 1995) and changes in protein tyrosine phosphorylation (Huang et al., 2005), is often used in the *in vitro* capacitation. However, it is of interest to note that BSA did not seem to be essential to guinea pig sperms capacitation in our experiment because absence of BSA did not influence neither hyperactivation nor acrosome reaction in our study. This could be attributed to the beneficial effect of PVA which is known to substitute for BSA and facilitate capacitation. But it did confirm that the absence of both BSA and PVA induced a significant decrease in protein tyrosine phosphorylation (Kulanand and Shivaji, 2001).

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