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Effects of Ca^{2+} and HCO_3^- on Capacitation, Hyperactivation and Protein Tyrosine Phosphorylation in Guinea Pig Spermatozoa

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ABSTRACT : In our previous report, we demonstrated that the tyrosine phosphorylation of sperm proteins (TPSP) of guinea pig was associated with capacitation and hyperactivation (CAHA), and Ca^{2+} and HCO_3^- were required for the initiation of CAHA and increasing the TPSP. The aim of this study was to further investigate the mechanism underlying the above events. The results showed that addition of cAMP agonists, dibutyryl-cAMP (db-cAMP) and isobutyl-methylxantine (IBMX), to HCO_3^--free medium significantly increased CAHA to the normal level (when sperm were incubated in TALP). Although addition of the cAMP agonists to Ca^{2+}-free medium increased CAHA, the percentages of hyperactivated and capacitated sperm were still significantly lower than the normal level. Compared with HCO_3^--free or Ca^{2+}-free medium, TPSP was increased when db-cAMP and IBMX were added in the media. H-89, a specific inhibitor of protein kinase A (PKA), inhibited CAHA in a dose-dependent manner and totally blocked TPSP. These results confirm a previous observation that Ca^{2+} and HCO_3^- regulated CAHA and TPSP in a cAMP/PKA pathway, and support an interation between TPSP and CAHA of sperm. Besides the cAMP/PKA pathway, Ca^{2+} might have also played a role in regulating CAHA by other pathways since the normal level of CAHA did not recover by adding cAMP agonists in the media. (**Key Words** : Capacitation, Hyperactivation, Tyrosine Phosphorylation, Sperm)

INTRODUCTION

Sperm capacitation in mammals has been showed to correlate with a series of changes including alterations in membrane fluidity (Harrison and Miller, 2000), cAMP concentration (Visconti et al., 1997, 1998), activation of PKA (Lefievert et al., 2002), acquisition of a special motility known as hyperactivation (Si and Okuno, 1999) and increase in protein tyrosine phosphorylation (Visconti et al., 1995b). However, molecular basis underlying these events is still not very clear. Capacitation can occur *in vitro* in defined media (Yanagimachi, 1994) whose compositions are based on the electrolyte concentration of oviductal fluid. It has been shown that certain components such as Ca^{2+}, HCO_3^- and BSA play an important role in the capacitation of sperm (Harayama and Kato, 2001).

In mammals, regulators of capacitation such as calcium, bicarbonate and BSA have different effects among species. For example, calcium, bicarbonate and BSA are necessary for the capacitation of mouse sperm (Visconti et al., 1995a) while BSA is not necessary for the capacitation of boar (Tardif et al., 2003) and ram sperm (Patricia Grasa et al., 2006). The increase of capacitation-associated protein tyrosine phosphorylation is not dependent on the presence of bicarbonate in hamster (Kulanand and Shivaji, 2001) and boar sperm (Tardif et al., 2003).

Extracellular calcium is not necessary for capacitation and protein tyrosine phosphorylation in human (Leclerc et al., 1998) and ram sperm (Patricia Grasa et al., 2006). Our previous study on guinea pig sperm capacitation showed that calcium and bicarbonate but not BSA were required for supporting *in vitro* capacitation and hyperactivation (Kong et al., 2008).

Tyrosine phosphorylation in sperm has been associated with hyperactivation (Si and Okuno, 1999) and capacitation (Visconti and Kopf, 1998). In mammals, tyrosine phosphorylation in sperm is regulated by cAMP because addition of cAMP agonists db-cAMP and IBMX increased tyrosine phosphorylation of sperm proteins (Visconti et al., 1995b; Leclerc et al., 1998). The effects of the cAMP agonists to enhance protein tyrosine phosphorylation appears to be at the level of the cAMP-dependent PKA, since a specific inhibitor of PKA (H-89) blocked the increases in protein tyrosine phosphorylation (Becky et al., 2004).

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Table 1. Composition of media used in various groups

Media	TALP	Ca^{2+}-free TALP	HCO_3^--free TALP	TALP add db-cAMP and IBMX	Ca^{2+}-free TALP add db-cAMP and IBMX	HCO_3^--free TALP add db-cAMP and IBMX	TALP with H-89 (different concentrations)
NaCl (114 mM)	+	+	+	+	+	+	+
KCl (3.16 mM)	+	+	+	+	+	+	+
$MgCl_2$ (0.35 mM)	+	+	+	+	+	+	+
$NaHCO_3$ (25.0 mM)	+	+	-	+	+	-	+
HEPES (25 mM)	-	-	+	-	-	+	-
Sodium pyruvate (0.25 mM)	+	+	+	+	+	+	+
Sodium lactate (12.5 mM)	+	+	+	+	+	+	+
Benzylpenicillin potassium (10,000 IU)	+	+	+	+	+	+	+
$CaCl_2$ (2.0 Mm)	+	-	+	+	-	+	+
BSA (3 mg/ml)	+	+	+	+	+	+	+
PVA (1 mg/ml)	+	+	+	+	+	+	+
db-cAMP (25 mM)	-	-	-	+	+	+	-
IBMX (100 μM)							
H-89 (1 μM, 3 μM, 5 μM, 10 μM)	-	-	-	-	-	-	+

In this study, we evaluated the effects of cAMP agonists and PKA specific inhibitor on capacitation, hyperactivation and protein tyrosine phosphorylation in guinea pig sperm to examine whether Ca^{2+} and HCO_3^- regulate capacitation, hyperactivation and protein tyrosine phosphorylation in a cAMP/PKA pathway.

MATERIALS AND METHODS

Sperm and media preparation

Caudal epididymal sperms were collected from mature guinea pig and diluted to 10^8 sperms per ml in media using the method described previously (Kong et al., 2008; Anil et al., 2006). The basic medium for the sperm was a modified TALP (Tyrode's albumin lactate pyruvate) medium, as described by Jha (Jha et al., 2002). Sperms were incubated at 37°C in different media described in Table 1. As a control, sperms were incubated in TALP medium. The pH of the media was adjusted to 7.8 using NaOH.

TALP was a complete medium; Ca^{2+}-free TALP was a medium without $CaCl_2$; HCO_3^--free TALP was a medium without $NaHCO_3$ and added HEPES to maintain the pH; the three media with db-cAMP and IBMX; TALP with different concentrations of H-89.

Solution of db-cAMP (Sigma, D0627) was made in ultrapure water. H-89 (Sigma, B1427) and IBMX (Sigma, I5879) were diluted in DMSO as stock solutions and added to the media to obtain the required concentrations, with DMSO<1% final solution.

Capacitation and hyperactivation

The CTC (Chlortetracycline) method (Pietrobon et al., 2001) was used to assess the capacitated state of the sperm. 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. 20 μl of sperm suspension from each condition was mixed with 20 μl of CTC. After 20 seconds, sperms were fixed by the addition of 3.2 μl of 12.5% glutaraldehyde in PBS (20 mM phosphate buffer, 150 mM NaCl pH 7.4). 20 μl mixture were placed on a clear slide. Sperms 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: i) pattern 'B' which is indicative of capacitation was characterized by head fluorescence with a fluorescence-free band in the post acrosomal region; ii) sperm either with a weakly homogeneous fluorescence or absence of fluorescence was characteristic of pattern 'AR' which represents acrosome-reacted cells; iii) the whole sperm with homogeneous fluorescence was referred as pattern 'F' represents non-capacitation.

Hyperactivation is a special type of sperm motility, which is characterised by high amplitude, asymmetrical beating pattern of the sperm tail. Hyperactivation was assessed in motile guinea pig sperm according to the procedure described by Zhang (Zhang et al., 2000). Hyperactivated sperm showed helical and circular motility patterns, unlike non-hyperactivated sperm which showed a planar motility pattern. Sperms were assessed using a phase-contrast microscopy (×400). We distinguished hyperactivated sperms from non-hyperactivated sperms by checking at motility pattern. For each treatment, three random fields were analyzed and the percentage of hyperactivated sperms was obtained. 100 sperms were

Table 2. Effects of cAMP agonists db-cAMP and IBMX on hyperactivation and capacitation of caudal epididymal guinea pig sperms

Media	Hyperactivation (%)	Pattern B (%)
TALP	63.67±2.31^{ae}	66.33±2.64^{a}
TALP add db-cAMP plus IBMX	79.00±3.61^{b}	71.00±3.21^{a}
TALP minus Ca^{2+}	3.67±1.63^{c}	16.67±2.08^{b}
TALP minus Ca^{2+} add db-cAMP plus IBMX	39.67±2.08^{d}	38.33±3.06^{c}
TALP minus HCO_3^-	3.33±2.53^{a}	26.33±3.51^{d}
TALP minus HCO_3^- add db-cAMP plus IBMX	62.00±2.37^{e}	63.00±4.58^{a}

Sperms were incubated in TALP (containing 2.5 mM $NaHCO_3$ and 2.0 mM $CaCl_2$), Ca^{2+}-free TALP, HCO_3^--free TALP media with or without 1 mM db-cAMP and 100 μM IBMX.

Different letters indicate significant differences between treatments, $p<0.01$.

scored for each point.

SDS-PAGE and western blotting

After incubation sperms were washed twice in PBS and suspended in Laemmli sample buffer (Luconi et al., 2005) and then 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. The expression of tyrosine-phosphorylated proteins was assessed by western blotting according to Kong (Kong et al., 2008). Net intensity was analyzed by Kodak 1 D Image Analytical System.

Stripping PVDF (polyvinylidene fluoride) membranes

The phosphotyrosine proteins were stripped and re-probed with an antibody against α-tubulin as described (Baker et al., 2004).

Statistical analysis

Results were presented as mean±SD of the number of samples indicated in each case. For each set of experiments, sperm samples were from at least three guinea pigs, and each experiment was repeated three times. To determine whether there were significant differences between treatments, LSD test was used after One-Way ANOVA.

RESULTS

Effects of cAMP agonists on hyperactivation and capacitation of guinea pig sperm

Sperms incubated in the medium without HCO_3^- or Ca^{2+} for 7 h showed a significant decline in hyperactivation and capacitation. Upon addition of 1 mM db-cAMP plus 100 μM IBMX to the HCO_3^--free medium, the percentages of hyperactivated sperms and pattern 'B' sperms were significantly increased and recovered to the normal level (incubated in the TALP medium). Addition of 1mM db-cAMP plus 100 μM IBMX to Ca^{2+}-free medium improved the percentages of hyperactivated sperms and pattern 'B' sperms, but the percentages were still significantly lower than the normal level.

When compared with the TALP medium, sperms incubated in the medium containing 1 mM db-cAMP plus 100 μM IBMX showed a significant increase in hyperactivation but the increase in capacitation was not significant (Table 2).

Effects of cAMP agonists substituted for Ca^{2+} or HCO_3^- on protein tyrosine phosphoryration

The absence of HCO_3^- or Ca^{2+} in TALP medium caused a decrease in protein tyrosine phosphorylation. However, addition of cAMP agonists, db-cAMP and IBMX, could promote the protein tyrosine phosphorylation (Figure 1A, B). Densitometric analyses showed that the expression of tyrosine-phosphorylated proteins at the 80, 45, 40 kDa molecular weight were increased and recovered to the normal level at 7 h when sperms were incubated in these media containing 1 mM db-cAMP and 100 μM IBMX. Addition of db-cAMP and IBMX also improved the level of protein tyrosine phosphorylation when sperms were incubated in the complete medium (Figure 1C). Sperms incubated for 1 h in complete medium did not display the 45 kDa tyrosine-phosphorylated protein, however, the protein was observed when sperm were incubated for 1 h in the medium containing db-cAMP and IBMX (Figure 1C).

Effect of PKA specific inhibitor H-89 on capacitation, hyperactivation and protein tyrosine phosphorylation

The percentage of hyperactivated sperms was significantly reduced when sperm samples were incubated for 7 h in TALP media containing different concentrations of H-89 (Table 3). The CTC pattern showed a slight decrease in the percentage of pattern 'B' sperms incubated in TALP medium containing 0.1 μM H-89. However, the percentage of pattern 'B' was significantly decreased in the presence of higher concentrations of H-89 (1 μM, 3 μM, 5 μM, 10 μM; Table 3). A significant decrease in tyrosine-phosphorylated proteins at the 40, 45, 80 kDa molecular weight was observed in the presence of 0.1 μM H-89. Only the 40 kDa tyrosine-phosphorylated protein was observed in

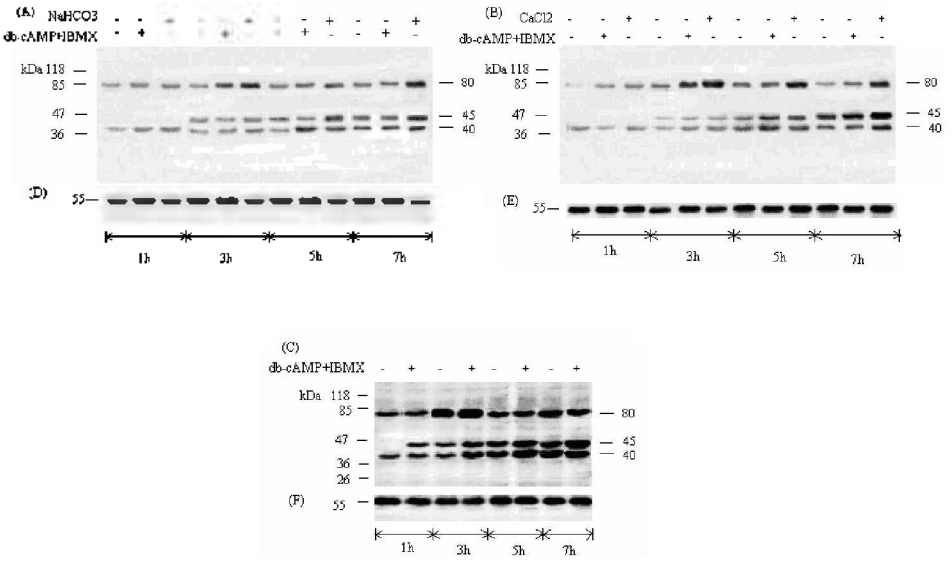

Figure 1. Effects of cAMP agonists on the appearance of tyrosine-phosphorylated proteins of caudal epididymal guinea pig sperms. Figure A, B and C indicate protein tyrosine phosphorylation of sperms incubated in HCO_3^--free TALP, Ca^{2+}-free TALP, and TALP media (containing 2.5 mM $NaHCO_3$ and 2.0 mM $CaCl_2$) with (+) or without (-) 1 mM db-cAMP and 100 μM IBMX. Figure D, E and F indicate the expression of α-tubulin in each total protein. Each experiment was repeated three times and a representative experiment is shown. The figures on the left show molecular weight of the pre-stained marker proteins and the figures on the right indicate molecular weight of the phosphorylated proteins.

the presence of 1 μM H-89. There was no tyrosine-phosphorylated protein detected in the presence of H-89 at higher concentrations (3 μM, 5 μM, 10 μM; Figure 2).

DISCUSSION

Results in this study showed that the percentages of hyperactivated (Table 2) and capacitated sperms (Table 2) were significantly increased and recovered to normal level (in TALP medium) at 7 h by addition of cAMP agonists db-cAMP and IBMX to the HCO_3^--free media. The protein tyrosine phosphorylation was restored with the same treatment (Figure 1A). These results are consistent with those reported in mouse (Visconti et al., 1995b) and ram (Patricia Grasa et al., 2006) showing that cAMP agonists could substitute for HCO_3^- in supporting capacitation, hyperactivation and protein tyrosine phosphorylation, and demonstrated that bicarbonate regulates capacitation, hyperactivation and protein tyrosine phosphorylation in a cAMP-dependent pathway.

The general standpoint is that Ca^{2+} plays an important part in capacitation and hyperactivation. But, previous results about its role in protein tyrosine phosphorylation are controversial. Some papers showed that Ca^{2+} stimulated protein tyrosine phosphorylation (Tardif et al., 2003) whereas others (Luconi et al., 1996) demonstrated that Ca^{2+} had an negative effect on protein tyrosine phosphorylation in human. Protein tyrosine phosphorylation was independent of Ca^{2+} in hamster (Kulanand and Shivaji, 2001) and ram sperms (Patricia Grasa et al., 2006). Our results showed that Ca^{2+} was a necessary factor in capacitation and hyperactivation and in increasing protein tyrosine phosphorylation in guinea pig sperm. The percentages of hyperactivated sperms and CTC pattern 'B'

Table 3. Effect of the protein kinase A inhibitor, H-89, on hyperactivation and capacitation

Media	Hyperactivation %	Pattern B %
TALP	63.00±3.51[a]	66.33±3.52[a]
TALP with 0.1 μM H-89	40.67±2.08[b]	60.67±2.31[a]
TALP with 1 μM H-89	18.00±2.76[c]	43.00±1.79[b]
TALP with 3 μM H-89	14.67±0.58[c]	27.67±1.16[c]
TALP with 5 μM H-89	9.33±1.53[d]	16.33±1.53[d]
TALP with 10 μM H-89	2.67±1.16[e]	9.00±2.25[e]

Sperms were incubated in TALP (containing 2.5 mM $NaHCO_3$ and 2.0 mM $CaCl_2$) with or without different concentrations of H-89 (0.1 μM, 1 μM, 3 μM, 5 μM, 10 μM).

Different letters indicate significant differences between treatments, $p<0.01$.

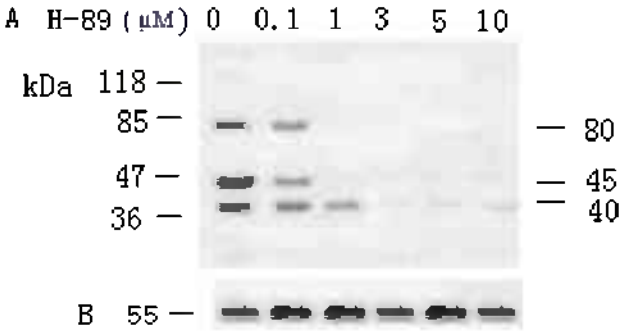

Figure 2. Effect of H-89 on the appearance of tyrosine-phosphorylated proteins of caudal epididymal guinea pig sperms. Figure A represents protein tyrosine phosphorylation of sperms incubated in TALP medium with or without increasing concentrations of H-89. Figure B shows the expression of α-tubulin in each total protein. Each experiment was repeated three times and a representative experiment is shown.

sperms, incubated in Ca^{2+}-free medium with cAMP agonists db-cAMP and IBMX, were increased but still significantly lower than the normal level (Table 2). These results were different from the report on mouse sperm which showed that the level of capacitation was restored (Visconti et al., 1995b). The protein tyrosine phosphorylation appeared to be completely restored with the same treatment (Figure 1B). We propose a hypothesis that, besides cAMP-dependent pathway, Ca^{2+} might regulate hyperactivation and capacitation by other signaling pathways since addition of cAMP agonists did not restore the level of hyperactivation and capacitation when sperms were incubated in Ca^{2+}-free medium.

Several studies implicated that inhibition of PKA activity inhibited protein tyrosine phosphorylation and capacitation significantly (Visconti et al., 1995b; Hannah et al., 1997). However, recent studies in human (Bajpai et al., 2003) and ram (Patricia Grasa et al., 2006) sperms showed that H-89, the specific inhibitor of PKA, inhibited protein tyrosine phosphorylation slightly. To examine the functions of PKA in capacitation, hyperactivation and protein tyrosine phosphorylation in guinea pig sperms, we assessed effects of PKA inhibitor H-89. Our results showed that H-89 at high concentrations (3 μM, 5 μM, 10 μM) totally inhibited protein tyrosine phosphorylation (Figure 2) although the concentration was lower than what was reported in bovine sperm (Hannah et al., 1997) which might be due to the specific difference in species. H-89 also caused a significant decrease in capacitation and hyperactivation (Table 3). These results indicate that PKA is involved in the signaling pathway regulating capacitation, hyperactivation and protein tyrosine phosphorylation, which further confirm the correlation between protein tyrosine phosphorylation and capacitation and hyperactivation.

In conclusion, our results demonstrated that Ca^{2+} and HCO_3^- regulated capacitation, hyperactivation and protein tyrosine phosphorylation in a cAMP/PKA pathway, and the correlation between protein tyrosine phosphorylation and capacitation, hyperactivation was further confirmed. Besides the cAMP/PKA pathway, Ca^{2+} might regulate capacitation and hyperactivation by other pathways since addition of cAMP agonists did not recover to the normal level of capacitation and hyperactivation.

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