

FSCB phosphorylation in mouse spermatozoa capacitation

Shun-Li Liu^{1, #}, Bing Ni^{2, #}, Xiang-Wei Wang³, Wen-Qian Huo¹, Jun Zhang¹, Zhi-Qiang Tian², Ze-Min Huang², Yi Tian², Jun Tang², Yan-Hua Zheng², Feng-Shuo Jin^{1, *} & Yan-Feng Li^{1, *}

¹Department of Urology, Daping Hospital, The Third Military Medical University, Chongqing 400042, ²Institute of Immunology PLA, Third Military Medical University, Chongqing 400038, ³Department of Urology, Xinqiao Hospital, The Third Military Medical University, Chongqing 400038, China

It is generally accepted that spermatozoa capacitation is associated with protein kinase A-mediated tyrosine phosphorylation. In our previous study, we identified the fibrous sheath CABYR binding protein (FSCB), which was phosphorylated by PKA. However, the phosphorylation status of FSCB protein during spermatozoa capacitation should be further investigated. To this aim, in this study, we found that phosphorylation of this 270-kDa protein occurred as early as 1 min after mouse spermatozoa capacitation, which increased over time and remained stable after 60 min. Immunoprecipitation assays demonstrated that the tyrosine and Ser/Thr phosphorylation of FSCB occurred during spermatozoa capacitation. The extent of phosphorylation and was closely associated with the PKA activity and spermatozoa motility characteristics. FSCB phosphorylation could be induced by PKA agonist DB-cAMP, but was blocked by PKA antagonist H-89. Therefore, FSCB contributes to spermatozoa capacitation in a tyrosine-phosphorylated format, which may help in further elucidating the molecular mechanism of spermatozoa capacitation. [BMB reports 2011; 44(8): 541-546]

INTRODUCTION

Spermatozoa freshly ejaculated into the female reproductive tract undergo an array of biochemical changes to have fertilizing capacity, a process called spermatozoa capacitation (1). Tyrosine phosphorylation is an important post-translational event and a hallmark of spermatozoa capacitation.

The spermatozoa tail fibrous sheath functions as an important mechanical component of flagella, and also serves as a scaffold for both glycolytic enzymes that provide energy for sperm motility and for constituents of signaling cascades (2).

*Corresponding authors. Feng-Shuo Jin, Tel: 86-23-68757949; Fax: 86-23-68757949; E-mail: jinf5505@163.com; Yan-Feng Li, Tel: 86-23-68757947; Fax: 86-23-68757947; E-mail: lyf1000@yahoo.com
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[#]These authors contributed equally to this work.

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There are more than 20 proteins associated with the fibrous sheath, including AKAP3, AKAP4 (3), TAKAP-80, GAPDS, HK1-S, GSK3 β , ALDOA, LDHA, SFEC, triose phosphate isomerase, GAPDH, pyruvate kinase, LDH-C, sorbitol dehydrogenase, GSTM5, FS39, Ropporin, Rhophilin, SP17, PDE4A, FSIP1 and FSIP2, ASP, and CABYR (4, 5). Nevertheless, the substrates and mechanism(s) of action of PKA in spermatozoa capacitation are still unknown.

Herein we describe findings that indicate FSCB is a novel protein expressed specifically on the surface of the fibrous sheath of the mouse spermatozoa principal piece (6). FSCB is a calcium-binding protein, which can be phosphorylated by PKA *in vitro*. As FSCB is found in proximity to other spermatozoa fibrous sheath proteins such as AKAP3, AKAP4 and CABYR and is capable of being phosphorylated and binding to calcium, it may be an important protein contributing to spermatozoa flagellar movement, spermatozoa capacitation and hyperactivation.

The present study was aimed at exploring the basic biological properties of FSCB and whether it is phosphorylated by PKA during spermatozoa capacitation, and the effect of PKA agonist DB-cAMP and PKA antagonist H-89 on FSCB phosphorylation and spermatozoa motility characteristics, in order to elucidate the molecular basis and regulatory mechanism(s) of spermatozoa capacitation.

RESULTS

Serine/threonine and tyrosine phosphorylation of FSCB during spermatozoa capacitation

We reported previously that FSCB is phosphorylated *in vitro* by the PKA catalytic subunit, and that it possesses multiple phosphorylation sites (6). To further investigate whether FSCB is phosphorylated during spermatozoa capacitation, spermatozoa before and at 2 h after capacitation were lysed and analyzed by Western blotting. The results indicated a visible 270 kDa positive band at 2 h after capacitation, suggesting that a 270 kDa protein can be phosphorylated by PKA. This positive band did not appear before capacitation (Fig. 1A), or in the negative control (Fig. 1B). To investigate whether this 270 kDa protein is just the FSCB protein, immunoprecipitation and Western blotting assays were conducted. Mouse spermatozoa before and at 2 h after capacitation were lysed and subjected to immunoprecipitation with the

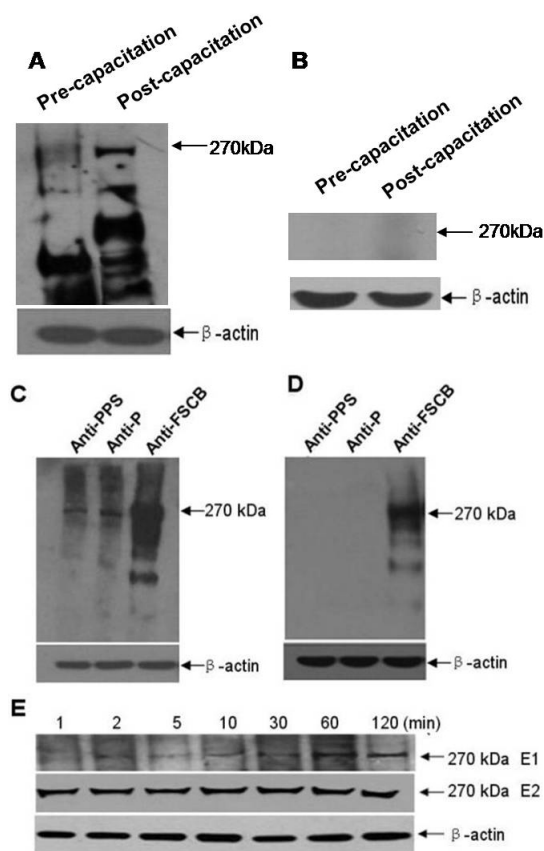


Fig. 1. FSCB phosphorylation during spermatozoa capacitation. Panels A and B, Western-blot analysis of spermatozoa lysates by using anti-phospho-(Ser/Thr) PKA substrate antibody and rabbit preimmune sera, respectively. A 270 kDa protein that can be phosphorylated during spermatozoa capacitation is indicated. Panels C and D, Immunoprecipitation and Western-blot assays for lysates of spermatozoa post- and pre-capacitation, respectively. The spermatozoa lysates were immunoprecipitated with anti-phospho-(Ser/Thr) PKA substrate (Anti-PPS), anti-phosphotyrosine (Anti-P) and anti-FSCB antibodies, followed by Western-blot assays by using anti-FSCB antibody. Protein size markers are indicated. Panel E, Western blotting assay of the spermatozoa lysates at various time points after capacitation. E1 and E2, Western blotting with anti-phospho-(Ser/Thr) PKA substrate antibody and anti-FSCB antibody, respectively. β -actin was used as an internal control in all Western blotting assays.

use of anti-phospho-(Ser/Thr) PKA substrate, anti-phosphotyrosine and anti-FSCB antibodies, respectively, followed by Western blotting using anti-FSCB antibody. The results showed that there was a 270 kDa band at 2 h after capacitation in all three immunoprecipitation products (Fig. 1C). However, this band was only observed in the immunoprecipitation product of anti-FSCB antibody before capacitation (Fig. 1D). Thus, these findings further demonstrated that the proteins precipitated by anti-phospho-(Ser/Thr) PKA substrate and anti-phosphotyrosine antibodies after spermatozoa capacitation was just the FSCB protein.

Moreover, it is demonstrated that FSCB phosphorylation does not occur before capacitation because FSCB cannot be precipitated by anti-phospho-(Ser/Thr) PKA substrate and anti-phosphotyrosine antibodies (Fig. 1D). To further study FSCB phosphorylation over time, aliquots of spermatozoa were lysed at 1, 2, 5, 10, 30, 60 and 120 min after capacitation. The lysates were subjected to protein quantification and analyzed by Western blotting with anti-phospho-(Ser/Thr) PKA substrate and anti-FSCB antibodies, respectively. The results indicated that FSCB phosphorylation occurred as early as 1 min after spermatozoa capacitation, which increased over time and remained stable after 60 min (Fig. 1E).

Effect of PKA activity on FSCB phosphorylation

The above results indicated that FSCB could be phosphorylated by PKA during spermatozoa capacitation. To further elucidate the molecular basis and regulatory mechanism for FSCB phosphorylation during spermatozoa capacitation, we investigated how PKA activity affected FSCB phosphorylation by using the agonist and the antagonist of PKA. DB-cAMP can penetrate the cytomembrane and promote the PKA function in the cell (7); In contrast, H-89 suppresses spermatozoa capacitation by suppressing cAMP-dependent PKA activity (8). We treated spermatozoa with H-89 and DB-cAMP, respectively, and then observed the status of FSCB phosphorylation. Results showed that 0.5 μ M H-89 significantly decreased spermatozoa PKA activity, compared with HTF medium control ($P < 0.01$) (Fig. 2A). However, 2.5 mM DB-cAMP could markedly increase the spermatozoa PKA activity, which was significantly higher than that observed in the M2 medium control group ($P < 0.01$) (Fig. 2B).

Western blotting assays for spermatozoa lysates showed an obvious phosphorylated protein band at 270 kDa in the HTF medium group and the M₂ medium containing the 2.5 mM DB-cAMP group, in contrast to a faint phosphorylation protein band at 270 kDa in the M₂ medium group and the HTF medium containing a 0.5 μ M H-89 group (Fig. 2C). No phosphorylation protein band at 270 kDa was observed when using rabbit preimmune serum as primary antibody (Fig. 2D). All these results suggested that H-89 suppressed PKA activity and subsequent FSCB phosphorylation, and DB-cAMP enhanced PKA activity and subsequent FSCB phosphorylation.

FSCB phosphorylation was closely associated with spermatozoa motility

Tyrosine phosphorylation in specific proteins is a hallmark of spermatozoa capacitation, and it is associated with spermatozoa motility characteristics, e.g., speed and wobbling frequency. PKA activity can affect the status of FSCB phosphorylation. Further study on the effects of PKA activity and FSCB phosphorylation on spermatozoa motility was necessary. We analyzed spermatozoa motility characteristics at the same time points with PKA activity and FSCB phosphorylation assay. As shown in Fig. 3A, (A + B)% (% moving forward spermatozoa) was significantly higher in the HTF group than the HTF medium containing the 0.5 μ M H-89 group at 1, 5, 10, 20, 30 and

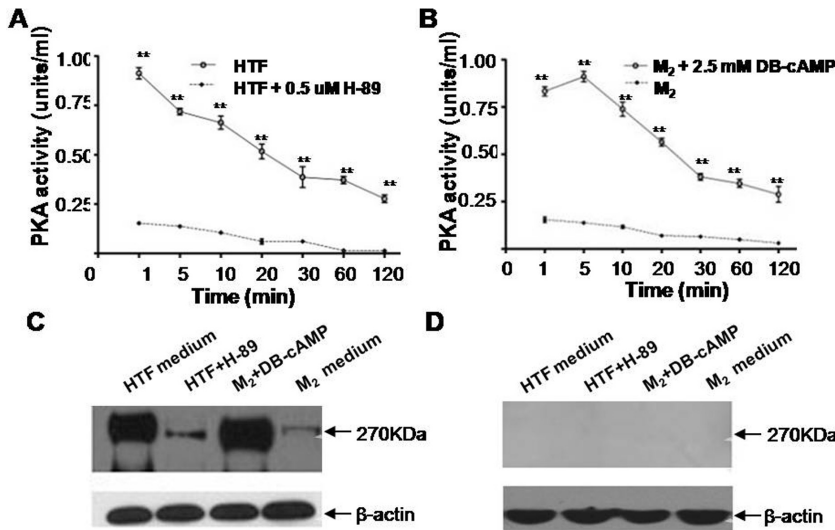


Fig. 2. Effect of PKA activity on FSCB phosphorylation. Mouse spermatozoa were cultured with HTF medium, HTF medium containing 0.5 μ M H-89, M2 medium, or M2 medium containing 2.5 mM DB-cAMP for the indicated time, the PKA activity and FSCB phosphorylation were detected at different time points. Panel A and B, the PKA activity of mouse spermatozoa after treatment with the indicated reagents for 1, 2, 5, 10, 30, 60 and 120 min. ** $P < 0.01$. Panel C and D, Western blotting analysis of the lysates of mouse spermatozoa treated with the indicated reagents for 30 min with anti-phosphorylation PKA substrate antibody (C) or rabbit preimmune serum (D).

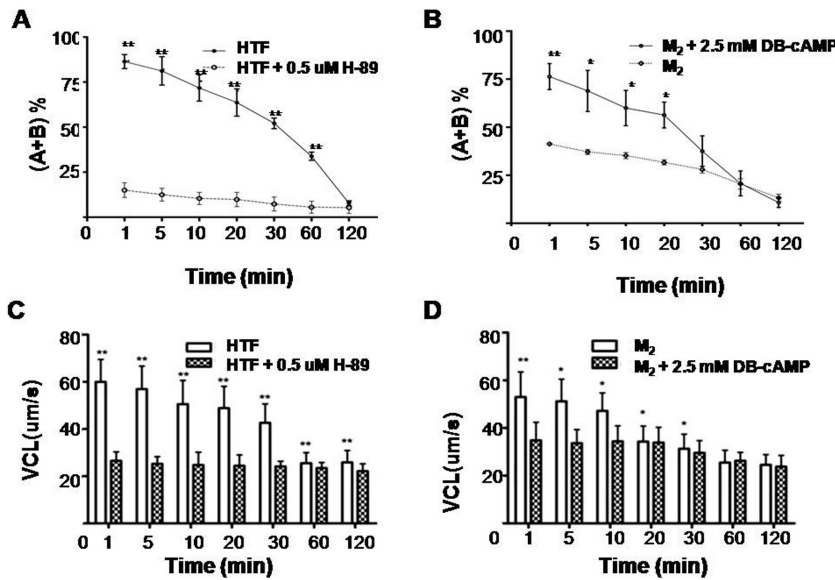


Fig. 3. PKA activity affects spermatozoa motility. Mouse spermatozoa were harvested and cultured with the indicated reagents for the indicated time after capacitation, followed by analysis for (A + B)% (percent of moving forward spermatozoa) and VCL (curvilinear velocity) by using Spermatozoa Class Analyzer 4.0. Panel A and B, effects of H-89 and DB-cAMP on spermatozoa (A + B)% at various time points, respectively. Panel C and D, effects of H-89 and DB-cAMP on spermatozoa VCL at various time points, respectively. * $P < 0.05$; ** $P < 0.01$.

60 min after capacitation ($P < 0.01$). In contrast, (A + B)% was higher in the M2 medium containing the 2.5 mM DB-cAMP group than the M2 group spermatozoa at 1, 5, 10 and 20 min after capacitation ($P < 0.05$) (Fig. 3B). Similarly, as shown in Fig. 3C and 3D, VCL (curvilinear velocity) was significantly lower in the HTF medium containing a 0.5 μ M H-89 group than in the HTF group at 1, 5, 10, 20, 30, 60 and 120 min after capacitation ($P < 0.01$), while it was higher in the M2 medium containing the 2.5 mM DB-cAMP group than in the M2 group at 1, 5, 10, 20 and 30 min after capacitation ($P < 0.05$). These results demonstrated that H-89 suppressed spermatozoa motility through suppression of PKA activity and phosphorylation of FSCB, and that Db-cAMP promoted such activities.

DISCUSSION

In the presence of BSA, Ca^{2+} , glucose, heparin, and HCO_3^- in media, spermatozoa membrane receptors change, leading to efflux of spermatozoa membrane cholesterol, membrane surface protein recombination, cAMP increase due to G-protein coupled activation of adenylate cyclase (AC), and PKA activity enhancement (9). Consequently, tyrosine kinase or phosphorylase is directly or indirectly regulated, and tyrosine phosphorylation occurs in certain proteins, which results in immediate biological effects (10, 11) and spermatozoa capacitation. However, the molecular mechanism involved in this process remains largely unknown (12). These proteins undergoing ty-

rosine phosphorylation distribute mainly to the spermatozoa tail, and they play important roles in regulating spermatozoa motility. Mouse spermatozoa with PKA catalytic subunit deficit cannot be capacitated because of tyrosine phosphorylation failure in certain proteins (13). FSCB is a recently discovered protein specifically expressed on the surface of the fibrous sheath of the mouse spermatozoa principal piece, and it binds CABYR (4). It was demonstrated that this protein can be phosphorylated by the PKA catalytic subunit *in vitro* and bind to calcium (2), suggesting its contribution to spermatozoa capacitation.

Anti-phospho-(Ser/Thr) PKA substrate antibody against the Arg-X-X- (Phospho-ser/thr) motif has been utilized in studying PKA-dependent ser/thr phosphorylation during spermatozoa capacitation (14, 15). Kiyoshi Miki *et al.* observed a 270 kDa band on a Western blot of post-capacitation spermatozoa lysates using anti-phospho-(Ser/Thr) PKA substrate antibody; however, they did not identify the protein (16). In the present study, we confirmed the presence of a 270 kDa protein band in post-capacitation spermatozoa lysates. Further study revealed the 270 kDa band in each lane loaded with lysates of spermatozoa at 120 min post-capacitation, however a positive band was observed in precapacitation spermatozoa lysates immunoprecipitated with anti-FSCB antibody only. These findings suggested that the 270 kDa protein just be FSCB and FSCB can be recognized and precipitated by anti-phospho-(Ser/Thr) PKA substrate and anti-phosphotyrosine antibodies, resulting in a 270 kDa band. Visconti *et al.* (17) found that the extent of tyrosine phosphorylation in certain proteins was closely associated with the phase of capacitation, i.e., the extent of phosphorylation increased over time. We observed that FSCB phosphorylation occurred within 1 min of culture with capacitating medium (HTF), and that phosphorylation levels were enhanced over time, and remained stable after 60 min. An in-depth investigation of FSCB will elucidate the molecular mechanism of spermatozoa capacitation.

The cAMP concentration influences ionic flux, motility characteristics and acrosome reaction of spermatozoa (9). After addition of Db-cAMP to a medium incapable of enabling hamster spermatozoa to capacitate, hamster spermatozoa can become capacitated (7). In contrast, after addition of H-89 to a medium capable of enabling spermatozoa to capacitate, tyrosine phosphorylation of related proteins and spermatozoa capacitation did not occur (8, 18), demonstrating that simple changes in the cAMP concentration also leads to changes in spermatozoa capacitation. In spermatozoa capacitation, PKA is the main target of cAMP. Changes in cAMP concentration may lead to changes in PKA activity and tyrosine phosphorylation (9). We added 0.5 μ M H-89 to the capacitating medium (HTF), and PKA activity was suppressed. By 30 min after sperm capacitation, Western blotting with anti-phospho-(Ser/Thr) PKA substrate antibody revealed a significant reduction in the 270 kDa band after addition of H-89, suggesting that PKA activity suppression reduced FSCB phosphorylation proportionally. After addition of 2.5 mM DB-cAMP to ordinary medium (M2),

PKA activity was increased significantly, and Western blotting with anti-phospho-(Ser/Thr) PKA substrate antibody revealed an enhanced 270 kDa band, suggesting that DB-cAMP enhanced PKA activity. An increase in PKA activity resulted in an enhancement of FSCB phosphorylation. Therefore, FSCB phosphorylation is closely influenced by PKA activity.

PKA catalyzed tyrosine phosphorylation in specific proteins is the molecular basis and important hallmark of spermatozoa capacitation (17, 18). Spermatozoa capacitation is characterized by acrosome reaction and spermatozoa hyperactivation (19), as well as fast, violent, whipping locomotion of the spermatozoa tail. Blazak *et al.* (20) asserted that quantitative analysis of spermatozoa motility and velocity is crucial for determining the deleterious effects in the reproductive system, and that the most sensitive measures are the percentage of motile cells, mean spermatozoa swimming speed, and linearity (LIN), as reported for rodents and humans (21, 22). We observed spermatozoa motility changes at numerous points up to 120 min post-capacitation. The results indicated that following addition of 0.5 μ M H-89 into the capacitating medium (HTF), (A + B)% motility parameters (e.g., VCL), the number of class A and class B spermatozoa decreased significantly. In the HTF group, parameters such as (A + B)% and VCL were the highest upon contact of HTF, and then decreased over time. The characteristics of the curves depicting these parameters were basically consistent with PKA activity changes, suggesting that spermatozoa motility characteristics are closely associated with PKA activity, and that FSCB contributes to spermatozoa capacitation.

Recent research on fibrous sheath-related proteins indicated that the fibrous sheath serves as a scaffold for multiple glycolytic enzymes and signaling cascade pathways, thus regulating spermatozoa motility. Moreover, the fibrous sheath is a regulator of motility of the spermatozoa tail. FSCB is a newly discovered CABYR-binding fibrous sheath protein that binds calcium and undergoes PKA-catalyzed tyrosine phosphorylation during spermatozoa capacitation. The extent of FSCB phosphorylation is closely associated with PKA activity and spermatozoa motility characteristics. Hence, FSCB contributes to spermatozoa capacitation. Further study of FSCB may help elucidate the molecular mechanism of spermatozoa capacitation and the search for potential contraceptive targets.

MATERIALS AND METHODS

Mice

Male Kunming mice (12-16 weeks old) were sacrificed and the epididymal end was cut as a whole, and was squeezed to the end by two tweezers. White mucilage was oozed out by using a syringe to cut the epididymis, which contained large amounts of spermatozoa and was transferred into a pre-equilibrated spermatozoa medium. In our research two kinds of spermatozoa medium were used. HTF medium (SAGE, USA) was a capacitation medium and M2 media (Millipore, Lot#00429-4) was a non-capacitation medium. All animals were maintained

in pathogen-free conditions. All of the animal studies were approved by the Institutional Animal Care and Use Committee at the Third Military Medical University, Chongqing, China.

Co-immunoprecipitation assay

Spermatozoa (approximately 2×10^8 cells/ml) were cultured in pre-equilibrated HTF and M2 media in a CO₂ incubator at 37°C for 2 h and lysed in 200 µl Celis buffer (6). FSCB is a high molecular weight protein with low solubility. Hence, the lysates need further processing to increase the solubility of FSCB. In brief, the lysates were added to a Millipore ultrafiltration tube to which 2 volumes of balanced solution (1,000 ml of 0.05 mol Tris-HCl solution, pH 8.5, 1 mmol EDTA, 1 mmol reduced glutathione, 0.1 mmol oxidized glutathione, 0.5 mol L-arginine. 0.15 mol NaCl) was added and mixed, followed by 35 min centrifugation at $6,000 \times g$. The procedure was repeated twice. The liquid in the ultrafiltration tube, i.e., post-ultrafiltration spermatozoa lysates, was transferred into an EP tube. 50 µl of Protein G (GE healthcare, 17-0618-01) was washed 3 times with 0.15 M NaCl, and centrifuged at $10,000 \times g$ for 1 min. The supernatants were discarded, and the sediments were incubated with the mixture of 10 µl of anti-phosphorylated PKA substrate antibody (Cell Signaling#9621), anti-FSCB antibody (homemade), anti-phosphotyrosine antibody (Millipore, #DAM1462605) and 480 µl of 0.15 M NaCl, respectively. The mixture was shaken for 90 min at room temperature, centrifuged at $10,000 \times g$ for 1 min, and washed 3 times with 0.15 M NaCl. Then, the sediments were added to 500 µl of 0.15 M NaCl containing 1 mg BS³ (Thermo Scientific, Lot#kDa132006), and shaken for 1 h at room temperature, followed by addition of 20 µl 1 M Tris.HCl pH 7.5, with a 30 min incubation at room temperature, then 1 min centrifugation at $10,000 \times g$. The sediments were washed 3 times with 0.15 M NaCl and washed once with a balanced solution. The supernatants were removed, and post-ultrafiltration spermatozoa lysates were added to the sediments. The mixture was shaken overnight at 4°C, then centrifuged for 1 min at $12,000 \times g$. The supernatants were removed, and the sediments were washed 3 times with a balanced solution, and centrifuged at $12,000 \times g$ for 1 min. The sediments were washed with 250 µl Celis buffer, and centrifuged at $12,000 \times g$ for 5 min. The final supernatants were used for Western-blot assay.

Western blotting

The above co-immunoprecipitation products with anti-phosphorylation PKA substrate, anti-FSCB, and anti-phosphotyrosine antibodies were quantified for total protein and subjected to 4-12% SDS-PAGE, followed by Western blot analysis with anti-FSCB antibody.

To investigate FSCB phosphorylation status at different time points after capacitation, mouse spermatozoa (2×10^8) in M₂ medium were vortexed gently to make spermatozoa disperse, and then were transferred into pre-equilibrated HTF medium for capacitation analysis. At 1, 2, 5, 10, 30, 60 and 120 min af-

ter capacitation, the same volume of spermatozoa was lysed, and the lysates were subjected to protein quantification and Western blotting with anti-phosphorylation PKA substrate and anti-FSCB antibodies.

To investigate the effect of PKA activity on FSCB phosphorylation, mouse spermatozoa were cultured for 30 min in a CO₂ incubator at 37°C with pre-equilibrated HTF medium, HTF medium containing 0.5 µM H-89, M₂ medium and M₂ medium containing 2.5 mM DB-cAMP. Same volume of spermatozoa was lysed, and the lysates were subjected to protein quantification and Western blotting assay with anti-phosphorylation PKA substrate antibody.

PKA activity assay

Mouse spermatozoa were cultured with HTF medium, HTF medium containing 0.5 µM H-89 (Merck#371963) M₂ medium, and M₂ medium containing 2.5 mM DB-cAMP (BIOLOG, GERMANY), respectively. Spermatozoa were cultured for 1, 2, 5, 10, 30, 60 and 120 min, whereupon an aliquot of the same volume of spermatozoa was centrifuged for 2 min at $12,000 \times g$. Spermatozoa were resuspended in 0.5 ml of ice-cold PKA extraction buffer (Promega, USA), and homogenized with an ice-cold homogenizer. The homogenates were centrifuged for 5 min at 4°C, $14,000 \times g$. A small quantity of cAMP-dependent protein kinase catalytic subunit was diluted with PKA dilution buffer to 2 µg/ml. After a 1 min incubation in 30°C water, the sample was added to the tube, followed by a 30-min incubation at room temperature, and a 10-min incubation at 95°C to terminate the reaction. 1 µl of 80% glycerol was added to each sample, mixed, then loaded onto 0.8% agarose gel, followed by a 15-18 min electrophoresis run at 100V until obvious band separation. The bands (approximately 250 µl in volume) were cut from the gel with a scalpel, and were melted at 95°C. 125 µl of hot agarose was transferred into an EP tube containing 75 µl melted gel and 50 µl glacial acetic acid, and the contents vortexed. Then, 250 µl of the solution was transferred to each well of a 96-well plate. After all samples were transferred to the plate, optical absorbance was measured at 570 nm. Agarose without short peptides was used as blank and the cAMP-dependent protein kinase catalytic subunit was used as positive control. The experiments were performed three times and the means of the results were calculated.

Spermatozoa motility assay

Mouse spermatozoa were cultured in HTF medium, HTF medium containing 0.5 µM H-89, M₂ medium, and M₂ medium containing 2.5 mM DB-cAMP. A 5 µl aliquot of spermatozoa was added to Microcells (Conception Technology), and analyzed at 37°C at 1, 2, 5, 10, 20, 30, 60 and 120 min after capacitation using a Spermatozoa Class Analyzer 4.0 (Spain). Spermatozoa motility was classified into the following groups: class A (moving forward quickly), class B (moving forward slowly), class C (wobbling in-place), and class D (not moving). In addition, the spermatozoa count, VCL (curvilinear velocity),

VSL (straight line velocity), VAP (average path velocity), ALH (amplitude of lateral head displacement), LIN (linearity), STR (straightness), WOB (wobble), and BCF (beat cross frequency) were measured. (A + B) % and VCL were deemed the key parameters in this study (21, 22).

Statistical analysis

Data were subjected to homoscedasticity analysis and *t* test.

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