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# Mechanisms Underlying the Inhibition of Murine Sperm Capacitation by the Seminal Protein, SPINKL

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## ABSTRACT

SPINKL, a serine protease inhibitor kazal-type-like protein initially found in mouse seminal vesicle secretions, possesses structurally conserved six-cysteine residues of the kazal-type serine protease inhibitor family. However, it has no inhibitory activity against serine proteases. Previously, it was found to have the ability to suppress murine sperm capacitation in vitro. Herein, we investigated the mechanisms underlying the suppressive effect of SPINKL on sperm capacitation. Three in vitro capacitation-enhancing agents, including bovine serum albumin (BSA), methyl-beta-cyclodextrin (MBCD), and dibutyryl cyclic AMP (dbcAMP), coupled with 3-isobutyl-1-methylxanthine (IBMX), were used to evaluate the influence of SPINKL on capacitation signaling. Preincubation of sperm with SPINKL suppressed BSA- and MBCD-induced sperm capacitation by blocking three upstream signals of capacitation that is the cholesterol efflux from sperm plasma membranes, extracellular calcium ion influx into sperm, and increases in intracellular cAMP. Moreover, SPINKL also inhibited downstream signal transduction of capacitation since it suppressed dbcAMP/IBMX and $N^6$-phenyl cAMP (6-Phe-cAMP)-activated cAMP-dependent protein kinase-associated protein tyrosine phosphorylation. Such inhibition is probably mediated by attenuation of SRC tyrosine kinase activity. Furthermore, SPINKL could not reverse capacitation once sperm had been capacitated by capacitation-enhancing agents or capacitated in vivo in the oviduct. SPINKL bound to sperm existed in the uterus but had disappeared from sperm in the oviduct during the sperm's transit through the female reproductive tract. Therefore, SPINKL may serve as an uncapacitation factor in the uterus to prevent sperm from precocious capacitation and the subsequent acrosome reaction and thus preserve the fertilization ability of sperm. J. Cell. Biochem. 114: 888–898, 2013. © 2012 Wiley Periodicals, Inc.

**KEY WORDS:** SERINE PROTEASE INHIBITOR; SPERM; CAPACITATION; cAMP; CHOLESTEROL; CALCIUM

Capacitation is a complex process first independently described and defined by Chang [1951] and Austin [1952]. It is a physiological change in sperm that occurs in the oviduct of some mammals to acquire the ability to fertilize an egg [Suarez, 2008]. It can be mimicked in vitro in specifically defined medium [Chang, 1951; Austin, 1952]. Our current knowledge of capacitation largely originates from in vitro studies [Visconti et al., 1995a; Cross, 1998; Visconti and Kopf, 1998; Naz and Rajesh, 2004; de Jonge, 2005].

Capacitation is initiated by removal of cholesterol from the sperm plasma membrane [Go and Wolf, 1985; Choi and Toyoda, 1998; Cross, 1998; Visconti et al., 1999a1999b; Shadan et al., 2004]. Cholesterol efflux leads to changes in the membrane structure and fluidity and increases in the permeability of sperm to calcium ($Ca^{2+}$) and bicarbonate ($HCO_3^-$) ions, thus raising levels of sperm intracellular calcium ions ($[Ca^{2+}]_i$) and the pH. Elevated levels of sperm intracellular $Ca^{2+}$ and $HCO_3^-$ can activate adenyl cyclase leading to increases in intracellular levels of cyclic(c)AMP,

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activation of cAMP-dependent protein kinase (PKA), and finally induction of tyrosine phosphorylation of a subset of sperm proteins [Visconti and Kopf, 1998].

The sperm's surface is immersed in a protein-rich solution of seminal plasma which is mixed from secretions of the accessory sexual glands. These seminal proteins interact with and modify changes in the sperm's physiology so that they can acquire the ability to fertilize that is capacitation [Yanagimachi, 1994]. Decapacitation factors, which cause capacitated sperm to lose the ability to fertilize, are present in seminal plasma [Chang, 1957]. They are removed from the surface of the sperm's head before or during the capacitation process. Potential decapacitation factors were characterized in seminal plasma, including semenogelin [de Lamirande et al., 2001], SVA [Huang et al., 2000], SVS2 [Kawano et al., 2008], SPINKL [Lin et al., 2008], and SERPINE2 [Lu et al., 2011], which are secreted by seminal vesicles, and the epididymis-secreted cysteine-rich secretary protein 1 [Roberts et al., 2003] and a 40-kDa glycoprotein [Fraser et al., 1990].

SPINKL, a serine protease inhibitor kazal-type-like protein initially identified from mouse seminal vesicle fluid, possesses structurally conserved six-cysteine residues of the kazal-type serine protease inhibitor family; even so, it has no inhibitory activity against common serine proteases such as trypsin, chymotrypsin, subtilisin, or elastase. Intriguingly, it can bind to sperm and suppress bovine serum albumin (BSA)-induced sperm capacitation and block sperm–oocyte interactions in vitro [Lin et al., 2008]. In this study, we investigated the mechanisms underlying the suppressive effect of SPINKL on sperm capacitation.

## MATERIALS AND METHODS

### ANIMALS

With the approval of the Animal Care and Use Committee of the Department of Medical Research, Mackay Memorial Hospital, specific pathogen-free outbreed ICR mice were treated according to Institutional Guidelines for the Care and Use of Experimental Animals, housed under controlled lighting (14 h of light and 10 h of dark) at 21–22°C and provided with water and NIH-31 laboratory chow ad libitum.

### PREPARATION OF SPINKL AND ITS ANTISERUM

Following a previously described method, the SPINKL protein was purified, and anti-SPINKL antiserum was prepared [Lin et al., 2008]. Preimmune rabbit antiserum was collected before immunization with the SPINKL antigen for control staining. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

### PREPARATION OF MOUSE SPERM

Epididymides were immediately removed after male mice (~14 weeks old) were sacrificed. The caudal epididymides were slit in prewarmed Biggers, Whitten, and Whittingham (BWW) media [Biggers et al., 1971] and incubated at 37°C in 5% (v/v) $CO_2$ for 15 min to allow motile sperm to swim upwards. The motile sperm in the upper layer were collected for analyses.

To isolate the ejaculated uterine and oviductal sperm, female mice (6 weeks old) were superovulated by an intraperitoneal injection of 10 IU of pregnant mare serum gonadotropin (Sigma-Aldrich, St. Louis, MO), followed by an intraperitoneal injection of 10 IU of human chorionic gonadotropin (China Chemical and Pharmaceutical, Hsinchu, Taiwan) 48 h later, and were subsequently mated with male mice (~16 weeks old). Female mice with plugged vaginas were sacrificed, and the ejaculated sperm in the uterine cavity were collected 3 h after copulation. In brief, the semen was repeatedly agitated by pipetting with phosphate-buffered saline (PBS). The sperm solution was washed three times by centrifuging at 100*g* for 10 min and resuspending it. Sperm were transferred onto slides, allowed to air dry, and then fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature.

When collecting sperm from the oviduct, there was an insufficient amount of sperm for analysis 1–2 h after intercourse. This study followed a previous report and collected sperm from the oviduct 3 h after mating since the majority of the sperm can be induced to undergo the acrosome reaction [Kawano and Yoshida, 2007]. Oviductal sperm were collected by flushing the oviduct with 37°C-prewarmed BWW medium on a hotplate set to that temperature. Sperm were transferred using a mouth pipette into an Eppendorf tube for the acrosome reaction analyses or onto slides and fixed in 4% (w/v) paraformaldehyde for subsequent immunostaining analyses.

### EVALUATION OF SPERM CAPACITATION AND THE ACROSOME REACTION

Capacitation-enhancing agents, including BSA, methyl-beta-cyclodextrin (MBCD), and cAMP analogs, such as dibutyryl cyclic AMP (dbcAMP), coupled with 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich), $N^6$-phenyl-cAMP (6-Phe-cAMP), and Sp-5,6-DCI-cBIMPS (BIMPS) (BIOLOG Life Science Institute, Bremen, Germany), are well known to induce sperm capacitation [Visconti et al., 1995ab, 1999a]. A chlortetracycline (CTC) fluorescence staining method was used to morphologically assess sperm capacitation [Ward and Storey, 1984; Lee and Storey, 1985]. Freshly prepared epididymal spermatozoa ($10^6$ cells/ml) were capacitated in 50 μl of BWW medium with BSA (3 mg/ml), MBCD (1 mM), or dbcAMP (1 mM) coupled with IBMX (100 μM) as the positive control, while the negative control contained no supplement, at 37°C in an atmosphere of 5% (v/v) $CO_2$ in humidified air for 90 min. To assess the effects of SPINKL on BSA-, MBCD-, and dbcAMP/IBMX-induced capacitation, 0.6 mg/ml of SPINKL, an effective dose as previously demonstrated [Lin et al., 2008], was preincubated with sperm for 15 min, and then these agents were individually added thereafter as described above. CTC staining of sperm was carried out following the original method and examined using a fluorescence microscope (BX40, Olympus, Tokyo, Japan).

Capacitation-accompanied increases in protein tyrosine phosphorylation of a subset of proteins were examined according to a previously described method [Visconti et al., 1995a]. In brief, about $5 \times 10^6$ spermatozoa/ml were incubated in modified Krebs-Ringer bicarbonate (HM) medium [Lee and Storey, 1986] with or without SPINKL (0.6 mg/ml) at 37°C for 15 min, and then BSA (3 mg/ml), MBCD (1 mM), dbcAMP (1 mM)/IBMX (100 μM), 6-Phe-cAMP

(1 mM), or BIMPS (1 mM) was individually added as described above for in vitro capacitation. After 90 min of incubation, the soluble fraction of sperm protein extracts was subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) on an 8% gel slab. Proteins on the gel were electrotransferred onto nitrocellulose paper. Western blot analyses were performed using an anti-phosphotyrosine antibody according to a previous method [Visconti et al., 1995a].

To detect the activity status of the SRC tyrosine kinase, a PhosphoDetect anti-SRC family ($pTyr^{416}$) antibody (Calbiochem, Merck Biosciences, Darmstadt, Germany) was used for Western blotting. Levels of α-tubulin protein detected by the anti-α-tubulin antibody (Sigma-Aldrich) were used to assess protein loading.

To examine if SPINKL could reverse in vivo-capacitated oviductal sperm to an uncapacitated state, freshly flushed oviductal sperm were or were not incubated with 0.6 mg/ml of SPINKL for 15 min and then treated with or without 5 μM A23187 (Sigma-Aldrich) in DMSO (0.1%) at 37°C for 30 min. Sperm were smeared on the slide and fixed with methanol for 30 s. The sperm acrosomal status was assessed by staining with 5 μg/ml of tetramethyl rhodamine isothiocyanate (TRITC)-conjugated peanut agglutinin lectin (PNA; Sigma-Aldrich) in the dark for 10 min and counterstaining with 5 μg/ml of Hoechst 33258. After three brief rinses with PBS, slides were mounted in 50 μl of ProLong Gold antifade medium (Invitrogen Molecular Probes, Eugene, OR) and immediately examined with a fluorescence microscope (BX40, Olympus).

### MEASUREMENT OF cAMP IN SPERM

Caudal epididymal spermatozoa ($10^6$ cells) cultured in HM medium were incubated with or without SPINKL (0.6 mg/ml) for 15 min at 37°C, followed by an additional 90-min incubation with BSA (3 mg/ml). After washing with PBS by centrifugation at 600*g* for 5 min, sperm were collected, and cAMP was extracted using the lysis buffer provided by the cAMP enzyme immunoassay kit (RPN225, GE Healthcare Life Sciences, Piscataway, NJ). The amount of cAMP in sperm was measured according to the manufacture's instructions.

### IMMUNOCYTOCHEMISTRY OF SPINKL ON SPERMATOZOA

To examine whether the SPINKL protein can bind to ejaculated sperm collected from the uterus or oviduct, freshly prepared sperm smeared on slides were fixed in 4% (w/v) paraformaldehyde, allowed to dry on a glass slide, and washed twice with PBS. After incubation in blocking solution consisting of PBS containing 10% (v/v) normal goat serum, for 1 h at room temperature, slides were incubated with anti-SPINKL antiserum or control rabbit antiserum at a dilution of 1:200 in blocking solution for 1 h. Slides were washed three times with PBS to remove excess antibodies before they were incubated with TRITC-conjugated goat anti-rabbit Immunoglobulin G (IgG; Jackson ImmunoResearch, West Grove, PA) diluted 1:100 in blocking solution for 40 min. All slides were then washed with PBS and counterstained with 5 μg/ml of Hoechst 33258. After three brief rinses with PBS, slides were mounted in 50 μl of ProLong Gold antifade medium (Invitrogen Molecular Probes) and photographed using an epifluorescence microscope (Olympus BX40) equipped with an Olympus DP-70 digital camera.

### CHOLESTEROL EFFLUX ASSAY

The cholesterol content was analyzed following a previously described protocol [Lu et al., 2011]. In brief, freshly prepared epididymal spermatozoa ($2 \times 10^6$ cells/ml) were preincubated with or without SPINKL (0.6 mg/ml) in 150 μl of BWW medium for 15 min and then capacitated with BSA (3 mg/ml) or MBCD (1 mM) at 37°C in an atmosphere of 5% (v/v) $CO_2$ in humidified air for 90 min. After incubation, sperm were centrifuged at 10,000*g* to separately collect the supernatant and sperm pellet. Samples were mixed with chloroform and methanol in a final ratio of chloroform:methanol:supernatant (or sperm) of 2:2:1.8. After vigorous vortexing, the mixture was centrifuged at 600*g* for 5 min, and the organic phase was transferred to a new Eppendorf tube and dried with a speed-vac. The cholesterol content was measured using an Amplex Red Cholesterol assay kit (Invitrogen Molecular Probes) according the manufacturer's instructions. To calculate the cholesterol content of the samples, a cholesterol standard curve was prepared using the cholesterol reference standard provided with the kit.

### MEASUREMENT OF SPERM INTRACELLULAR CALCIUM ION CONCENTRATION

Levels of $[Ca^{2+}]_i$ in sperm were evaluated by the fluorescence probe, Fluo-4 acetoxymethyl ester (Fluo-4 AM, Invitrogen Molecular Probes). Briefly, freshly prepared epididymal spermatozoa ($\sim 2 \times 10^6$ cells/ml) were incubated with 5 μM of Fluo-4 AM in calcium-free BWW medium at 37°C in the dark for 20 min under 5% (v/v) $CO_2$ in humidified air, and then sperm cells were washed twice with the same medium to remove free Fluo-4 AM. To examine the effect of SPINKL on the capacitation-induced calcium influx, Fluo-4 AM-loaded sperm were incubated in BWW medium treated with or without SPINKL (0.6 mg/ml) for 15 min at 37°C, followed by an additional 90-min incubation with BSA (3 mg/ml) or MBCD (1 mM) at 37°C with 5% (v/v) $CO_2$. After washing with PBS for 5 min at 600*g*, sperm $[Ca^{2+}]_i$ were analyzed by flow cytometry (FACScan; Becton Dickinson Biosciences, Mountain View, CA) or smeared on slides for fluorescence image analyses.

### STATISTICAL ANALYSIS

Data are presented as the mean ± SD. Differences were analyzed by a paired Student's *t*-test using GraphPad software (GraphPad, San Diego, CA). A $P$ value of $<0.05$ was considered significant.

## RESULTS

### SPINKL SUPPRESSED IN VITRO SPERM CAPACITATION INDUCED BY CAPACITATION-ENHANCING AGENTS

To confirm the suppressive effect of SPINKL on sperm capacitation, epididymal sperm preincubated with SPINKL were capacitated using well-defined capacitation-enhancing agents that is BSA, MBCD, or dbcAMP/IBMX, and assessed by CTC fluorescence staining. As shown in Figure 1, the majority of sperm remained uncapacitated in BWW medium without supplementation (control, gray bar). Supplementation of medium with SPINKL had no effects on the population of capacitated sperm (hatched bar). However, the population of capacitated sperm remarkably increased after supplementation with BSA, MBCD, or dbcAMP/IBMX (blank

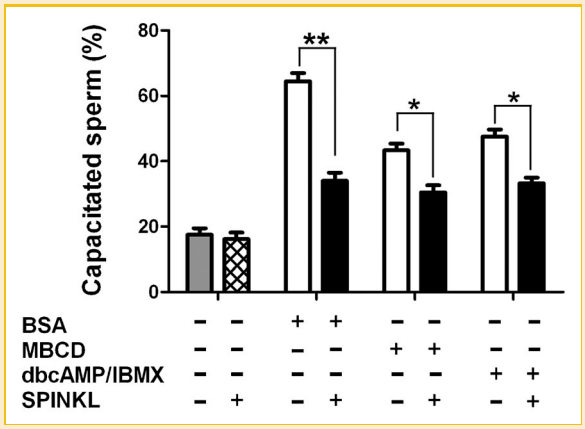


Fig. 1. SPINKL inhibited in vitro sperm capacitation as evaluated by a CTC fluorescence staining method. Epididymal sperm cultured in BWW medium were incubated with or without SPINKL (0.6 mg/ml) for 15 min and then were capacitated using three capacitation-enhancing agents, including BSA (3 mg/ml), MBCD (1 mM), and dbcAMP (1 mM), coupled with IBMX (100 μM) at 37°C for 90 min. CTC fluorescence staining was conducted to determine the population of capacitated sperm. A minimum of 200 sperm per trial was evaluated. Percentages of capacitated sperm under different experimental conditions without (gray bar and blank bars) or with SPINKL (0.6 mg/ml) (hatched bar and filled bars) are shown. Data are the mean ± SD from three independent experiments. Asterisks indicate significant differences compared with medium supplemented with BSA (**$P < 0.01$), MBCD (*$P < 0.05$), and dbcAMP/IBMX (*$P < 0.05$).

bars). Moreover, preincubation of sperm with SPINKL significantly inhibited sperm capacitation induced by the three agents in vitro (filled bars).

Next, we assessed tyrosine phosphorylation of the capacitation-associated sperm protein. As shown in Figure 2, limited proteins, with the exception of the BSA group (Fig. 2A), were tyrosine phosphorylated in the control experiment with no supplementation (lane 1). Phosphorylation was significantly enhanced when sperm were incubated in medium with any of the three agents (lane 2). However, when sperm were preincubated with SPINKL, phosphorylation was prominently inhibited regardless of the addition of any of the capacitation-enhancing agents (lane 3). Incubation with SPINKL even seemed to decrease the extent of sperm protein tyrosine phosphorylation compared with the control (lane 4). It was noted that the characteristic capacitation-associated protein tyrosine phosphorylation induced by BSA, MBCD, and dbcAMP/IBMX was prevented by prior action of SPINKL on sperm, indicating that SPINKL possesses an inhibitory effect on murine sperm capacitation in vitro.

## SPINKL COULD NOT REVERSE SPERM CAPACITATION IN VITRO OR IN VIVO

To determine if BSA-, MBCD-, or dbcAMP/IBMX-induced sperm capacitation could be reversed by SPINKL, SPINKL protein was supplemented after incubation with capacitation-enhancing agents for 30, 60, and 75 min. Results showed that SPINKL had an inhibitory effect only during early capacitation for 30 min by the three agents (Fig. 2, lane 5; cf. lanes 2 and 5). Once the sperm had been capacitated for 60 or 75 min, SPINKL showed no capability to reverse tyrosine phosphorylation of the sperm protein induced by the three agents (Fig. 2, lanes 6 and 7).

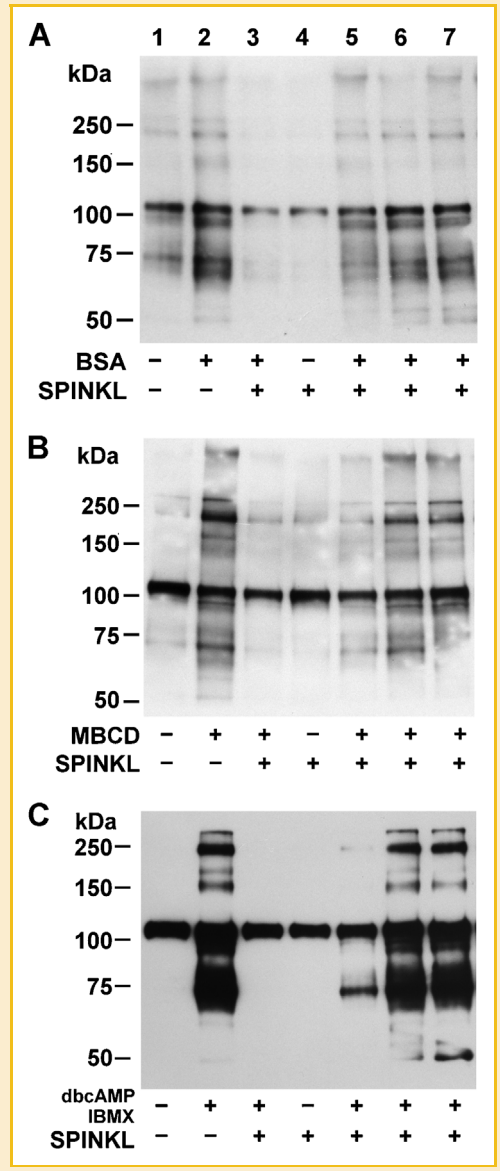


Fig. 2. SPINKL inhibited BSA-, MBCD-, and dbcAMP/IBMX-induced sperm protein tyrosine phosphorylation. Epididymal sperm cultured in HM medium were incubated with or without SPINKL (0.6 mg/ml) for 15 min and then were capacitated using three capacitation-enhancing agents, (A) BSA (3 mg/ml), (B) MBCD (1 mM), and (C) dbcAMP (1 mM), coupled with IBMX (100 μM) at 37°C for 90 min. Various incubations were carried out as follows: sperm incubated with medium only served as the negative control (lane 1) or medium supplemented with the capacitation agents as the positive control (lane 2); sperm incubated with SPINKL for 15 min and then supplemented with capacitation-enhancing agents for an additional 90 min (lane 3); sperm incubated with only SPINKL for 90 min (lane 4); medium supplemented with SPINKL after an enhancing agent had been incubated for 30 (lane 5), 60 (lane 6), and 75 min (lane 7), and coincubation was stopped at 90 min. The soluble fraction of the sperm lysate was resolved by SDS–PAGE, electrotransferred onto a nitrocellulose membrane, and immunoblotted with anti-phosphotyrosine antibodies.

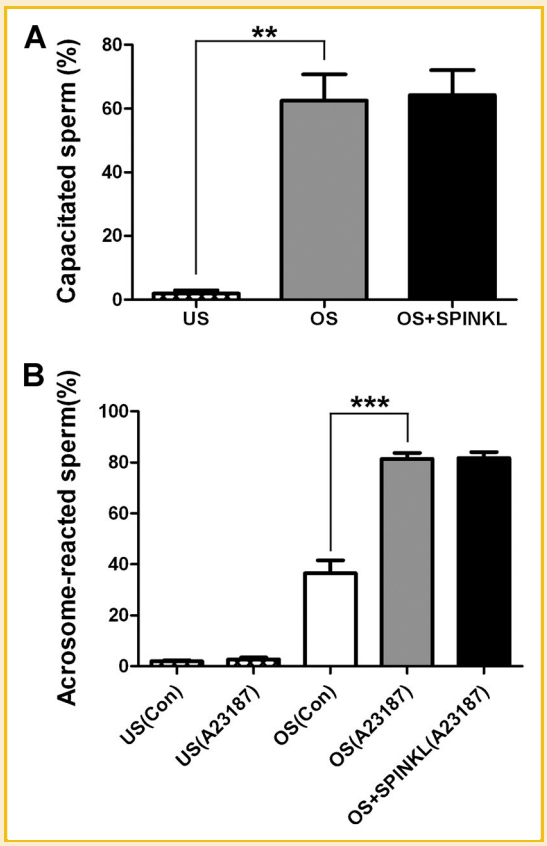


Fig. 3. No effects of SPINKL were found on capacitation or the acrosome reaction in ejaculated sperm. A: The capacitation states of ejaculated sperm collected from the uterus and oviduct were assessed by CTC fluorescence staining. Uterine sperm (US) and oviductal sperm (OS) were directly subjected to CTC fluorescence staining, or some OS were incubated with the SPINKL protein for 15 min and were then stained by CTC. B: The acrosome reaction induced using ionophore A23187 was evaluated by PNA-TRITC fluorescence staining. Sperm were incubated with or without the SPINKL protein for 15 min and then treated with or without A23187 (Con). A single animal was used for one group of experiments that is a portion of sperm was used for the capacitation assay and another portion was used for evaluating the acrosome reaction. A minimum of 50 sperm was counted for each assay. Data are the mean ± SD from eight independent experiments. Asterisks indicate significant differences compared with the control group ($^{**}P < 0.01$; $^{***}P < 0.001$).

To examine whether in vivo capacitation of sperm collected from the oviduct could be reversed by SPINKL, freshly flushed oviductal sperm were preincubated with SPINKL and then induced to undergo the acrosome reaction by the A23187 ionophore. We followed a previous report to collect sperm from the oviduct at 3 h after mating since about 60–70% of sperm can be induced to undergo the acrosome reaction at that time [Kawano and Yoshida, 2007]. As expected, over 60% or 80% of oviductal sperm had undergone capacitation (Fig. 3A, gray bar) or the acrosome reaction induced by the A23187 ionophore (Fig. 3B, gray bar), respectively, while vehicle only (0.1% DMSO) produced an acrosome reaction of about 40% which was similar to the control (no A23187 treatment, Fig. 3B, blank bar). In contrast, sperm collected from the uterus showed nearly no capacitation or acrosome reaction (Fig. 3A and B, hatched bars), which is consistent with a previous report [Kawano and Yoshida, 2007]. Prior incubation with SPINKL, however, had no influence on capacitation or the A23187-induced acrosome reaction (Fig. 3, filled bars). These findings suggest that SPINKL was unable to reverse sperm capacitation.

## SPINKL BOUND TO SPERM IN THE UTERUS BUT NOT IN THE OVIDUCT

Immunofluorescence staining was applied to determine the destination of SPINKL on ejaculated sperm during transit through the female reproductive tract. Sperm morphology was examined for comparison (Fig. 4a, c, e, and g). Sperm collected from the uterus showed a SPINKL protein signal on the tail compared with control staining (Fig. 4b and d); however, this staining had disappeared or was nearly undetectable on sperm isolated from the oviduct (Fig. 4d, f, and h).

## SPINKL PREVENTED CHOLESTEROL EFFLUX FROM CAPACITATED SPERM

To understand how SPINKL suppresses sperm capacitation induced by capacitation-enhancing agents, the cholesterol content of sperm plasma membranes was first examined, since cholesterol efflux is one of the initiating events of sperm capacitation. As shown in Figure 5A, elevation of cholesterol in the medium demonstrated BSA-promoted cholesterol release from murine sperm plasma membranes. MBCD caused an even greater cholesterol efflux, showing similar effects toward human sperm [Chiu et al., 2005]. However, the release was significantly suppressed by SPINKL. SPINKL itself showed no effects on the cholesterol content of sperm plasma membranes, and cholesterol levels of SPINKL-treated sperm remained nearly unchanged (Fig. 5B). Thus, its suppressive effect on sperm capacitation induced by the three capacitation-enhancing agents may initially have been caused by blocking cholesterol's efflux from sperm plasma membranes.

## SPINKL SUPPRESSED BSA- AND MBCD-INDUCED ELEVATION OF THE INTRACELLULAR CALCIUM ION CONCENTRATION IN MOUSE SPERM

Elevation of intracellular [$Ca^{2+}$] was shown to be associated with mouse sperm capacitation induced by BSA [Xia and Ren, 2009]. To examine whether the suppressive effect of SPINKL on BSA- and MBCD-induced sperm capacitation is caused by blocking $Ca^{2+}$ influx into sperm, a Fluo-4 AM fluorescence probe coupled with flow cytometry was used to measure $[Ca^{2+}]_i$ of sperm. As shown in Figure 6A, BSA and MBCD significantly increased $[Ca^{2+}]_i$ of sperm. However, SPINKL significantly reduced BSA- and MBCD-induced elevation of $[Ca^{2+}]_i$ but had little or no effect on basal $[Ca^{2+}]_i$ compared with control sperm. In contrast, fluorescence images of $[Ca^{2+}]_i$ in sperm under different treatments also showed similar results (Fig. 6B). BSA prominently enhanced the increase in $[Ca^{2+}]_i$ (cf. Fig. 6Ba and Bb). SPINKL itself had little effect on sperm $[Ca^{2+}]_i$ (cf. Fig. 6Ba and Bc), but obviously reduced the BSA-induced $[Ca^{2+}]_i$ signal (cf. Fig. 6Bb and Bd).

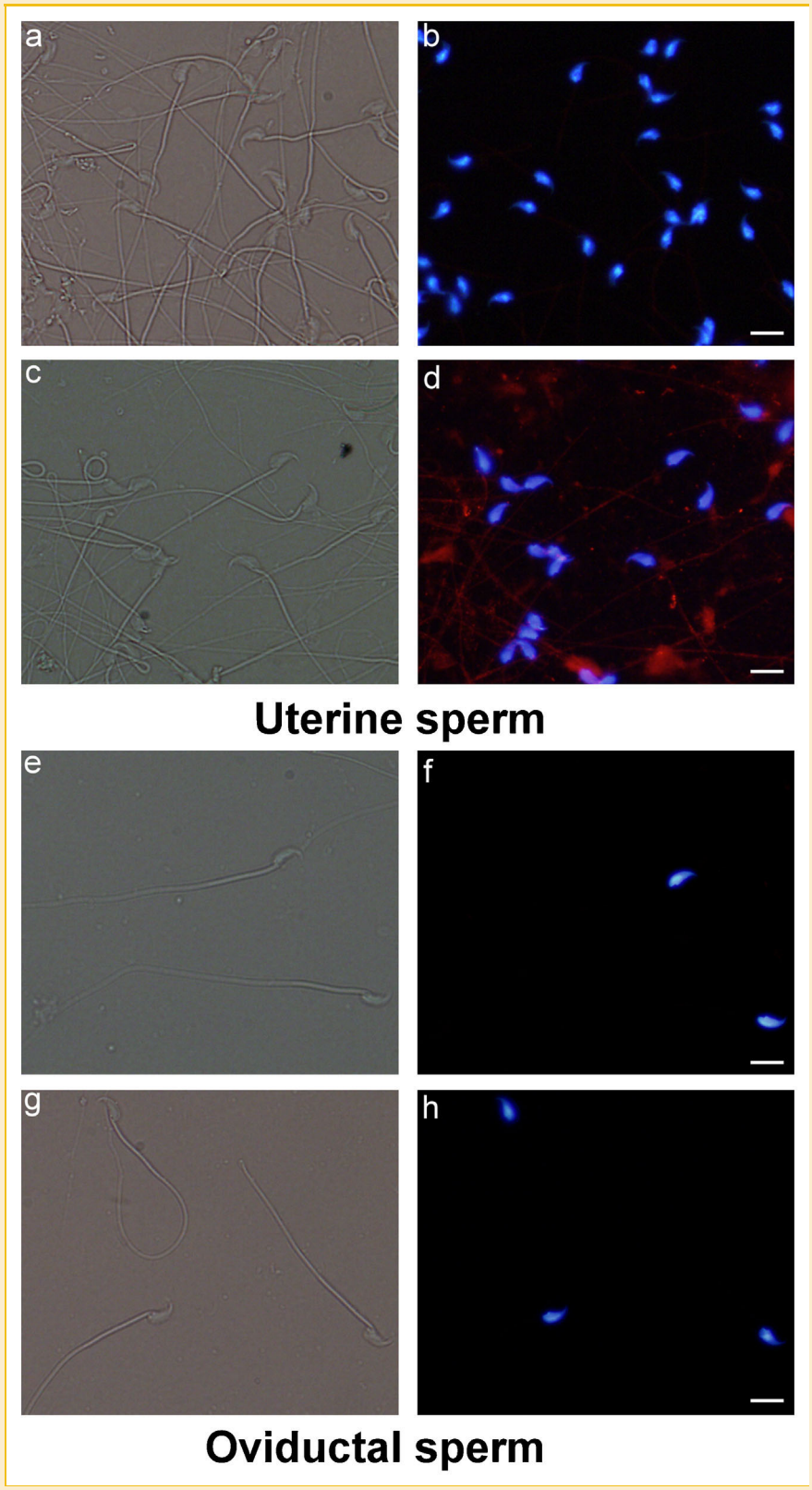


Fig. 4. Immunolocalization of SPINKL on the tail of ejaculated sperm. Ejaculated sperm collected from the uterus (a–d) and oviduct (e–h) were smeared on slides for immunolocalization staining for SPINKL. Phase-contrast images of sperm are shown to reveal the sperm morphology (a, c, e, g). Slides were incubated with control antiserum (b and f) and anti-SPINKL antiserum (d and h) at a dilution of 1:200, and then treated with TRITC-conjugated goat anti-rabbit IgG and counterstained with Hoechst dye to localize the nuclei for contrast. The SPINKL protein was detected on the tail region of sperm collected from the uterus (d) but not the oviduct (h). Bar = 20 μm.

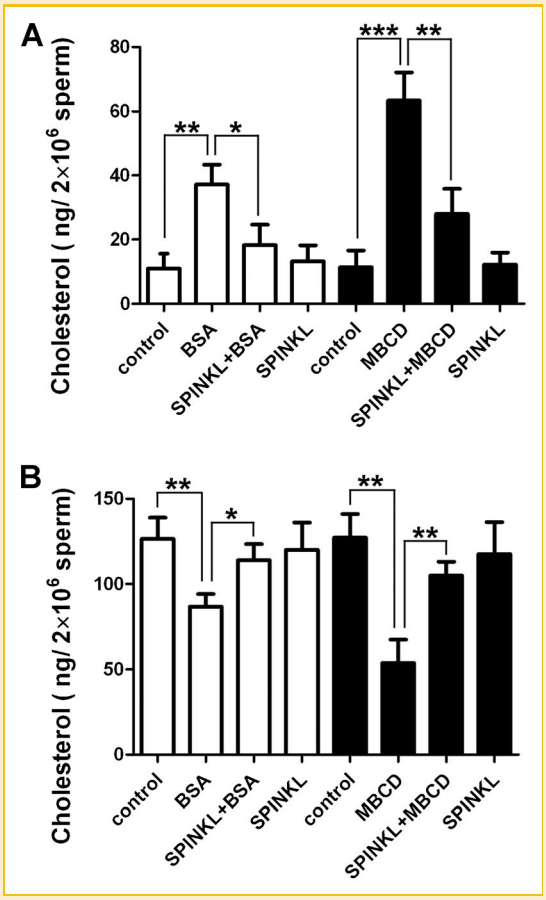


Fig. 5. SPINKL inhibited cholesterol efflux induced by capacitation-enhancing agents from capacitated sperm. Epididymal sperm cultured in BWW medium were incubated with or without SPINKL (0.6 mg/ml) for 15 min and then were capacitated by the capacitation inducers, BSA (3 mg/ml) or MBCD (1 mM), at 37°C for 90 min. After incubation, cholesterol was extracted from the medium (A) and sperm (B) by the procedure described in Materials and Methods Section. Data are the mean ± SD from three independent experiments. Asterisks indicate significant differences compared with medium supplemented with BSA ($^{*}P < 0.05$; $^{**}P < 0.01$) or MBCD ($^{**}P < 0.01$; $^{***}P < 0.001$).

### SPINKL ATTENUATED PKA-ACTIVATED DOWNSTREAM PROTEIN TYROSINE PHOSPHORYLATION

To assess the effects of SPINKL on changes in sperm's intracellular cAMP concentration ($[cAMP]_i$), epididymal sperm were incubated with or without SPINKL. As shown in Figure 7A, SPINKL itself showed no effects on sperm $[cAMP]_i$ compared with control sperm without SPINKL treatment (gray vs. blank bar). BSA initiates extracellular calcium influx and then activates adenyl cyclase [Hyne and Garbers, 1979], which is responsible for cAMP synthesis. Thus, as expected, BSA induced increases in sperm $[cAMP]_i$; however, such increases were suppressed by SPINKL (black vs. hatched bar).

To clearly define the suppressive effect of SPINKL on cAMP/PKA-associated sperm protein tyrosine phosphorylation, we evaluated the tyrosine phosphorylation pattern induced by cAMP-elevating agents, such as dbcAMP/IBMX, 6-Phe-cAMP, and BIMPS. As shown in Figure 7B–D, levels of sperm protein tyrosine phosphorylation were significantly enhanced by these cAMP analogs, while levels were prominently attenuated by the PKA inhibitor, H89, confirming that sperm protein tyrosine phosphorylation is primarily mediated by the cAMP/PKA pathway [Visconti et al., 1995b]. SPINKL suppressed dbcAMP/IBMX- and 6-Phe-cAMP-induced but only slightly inhibited BIMPS-induced sperm protein tyrosine phosphorylation.

A tyrosine kinase, SRC, was identified to be an intermediate PKA-activated tyrosine kinase, which drives downstream sperm tyrosine phosphorylation [Baker et al., 2006]. To assess if SPINKL had any effects on SRC kinase activity, a PKA-specific activator, 6-Phe-cAMP, was used to stimulate PKA-associated tyrosine phosphorylation signaling. As shown in Figure 7E, in sperm cultured with 6-Phe-cAMP, the SRC kinase produced phosphorylation of tyrosine residue 416, representing activation of the kinase [Baker et al., 2006]. Its tyrosine phosphorylation levels were obviously reduced when sperm were incubated with H89 or SPINKL. This finding indicates that the suppressive effect of SPINKL on cAMP/PKA-associated sperm protein tyrosine phosphorylation is probably mediated by inhibiting the activity of the SRC kinase.

## DISCUSSION

In this study, we revealed that the mouse seminal protein, SPINKL, inhibits BSA- and MBCD-induced sperm capacitation by blocking cholesterol efflux from sperm surface membranes and extracellular calcium influx into sperm. Moreover, SPINKL suppressed cAMP analog-activated PKA-associated sperm protein tyrosine phosphorylation probably by inactivating SRC, a PKA-activated downstream tyrosine kinase.

SPINKL itself had no effect on sperm endogenous $[cAMP]_i$, indicating that SPINKL does not affect the activity of adenyl cyclase. However, it inhibited BSA induced increases in intracellular cAMP concentration in sperm. This may have been caused by blocking the extracellular calcium influx into sperm, since calcium ion concentration surges can activate the activity of adenyl cyclase [Hyne and Garbers, 1979], thus leading to increases in $[cAMP]_i$.

The cAMP analogs, dbcAMP, 6-Phe-cAMP, and BIMPS, are membrane-permeable and are known to activate PKA activity [Ogreid et al., 1989; Sandberg et al., 1991]; they can bypass the early signaling of capacitation and directly activate PKA activity, and then PKA somehow activates downstream tyrosine kinases leading to increases in sperm protein tyrosine phosphorylation [Visconti et al., 1995b]. Sperm protein tyrosine phosphorylation is believed to be a late event in capacitation; however, this was inhibited by SPINKL. This finding prompted us to assess the effect of SPINKL on the activity of the tyrosine kinase downstream of PKA. In this study, tyrosine phosphorylation of sperm protein induced by the three cAMP analogs was suppressed by H89; however, SPINKL suppressed dbcAMP/IBMX- and 6-Phe-cAMP-induced but only slightly inhibited BIMPS-induced sperm protein tyrosine phosphorylation. This finding suggests that SPINKL might inhibit PKA-activated downstream tyrosine phosphorylation signaling by inactivating different intermediate tyrosine kinases.

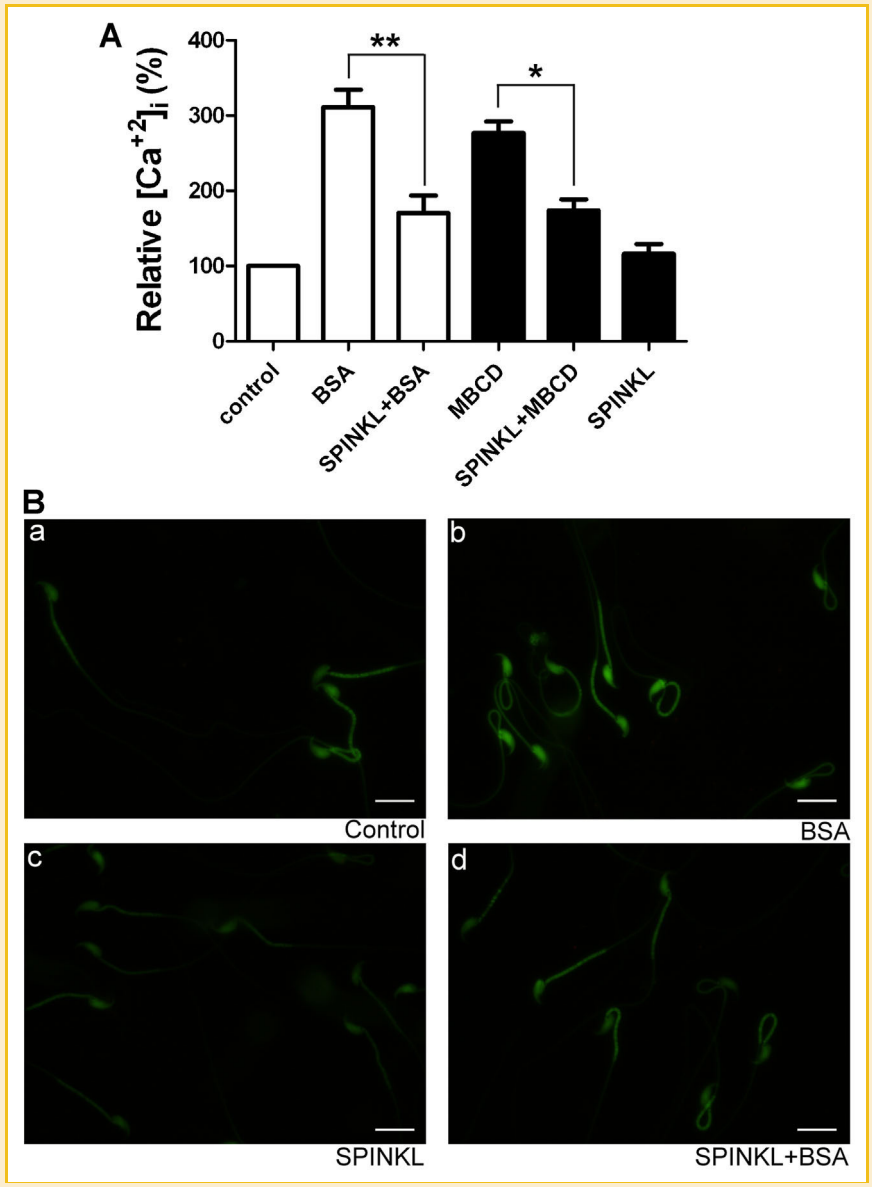


Fig. 6. SPINKL inhibited the elevation of $[Ca^{2+}]_i$ induced by capacitation-enhancing agents in mouse sperm. To detect sperm $[Ca^{2+}]_i$, sperm were preloaded with Fluo-4 AM. After washing, sperm were incubated with or without SPINKL (0.6 mg/ml) for 15 min, followed by incubation with BSA (3 mg/ml) or MBCD (1 mM) for an additional 90 min. $[Ca^{2+}]_i$ was measured by flow cytometry (A). Data are the mean ± SD from three independent experiments. Asterisks indicate significant differences compared with medium supplemented with BSA ($^{**}P < 0.01$) or MBCD ($^{*}P < 0.05$). Fluorescence images of sperm under various treatments (B): (a) control, (b) BSA (3 mg/ml), (c) SPINKL (0.6 mg/ml), and (d) coincubation with SPINKL and BSA.

SRC, a key tyrosine kinase activated by PKA, is essential for capacitation-associated sperm protein tyrosine phosphorylation. It was found to be associated with lipid rafts and localized on the midpiece of mouse sperm [Baker et al., 2006]. In this study, we found that the suppressive effect of SPINKL on cAMP/PKA-activated sperm protein tyrosine phosphorylation may attenuate the activity of the SRC kinase.

The exchange protein directly activated by cAMP (Epac) was recently found to be another downstream effector of cAMP [de Rooij et al., 1998; Kawasaki et al., 1998]. The cAMP analog, 8-(4-chlorophenylthio)-2′-o-methyl-cAMP (8-pCPT), can activate Epac but not PKA [Enserink et al., 2002]. To evaluate if SPINKL had any effects on Epac activity, sperm were cultured in HM medium without capacitation-enhancing agents, and 8-pCPT (50 μM) was added in the presence of SPINKL. The results showed that Epac had no effects on sperm protein tyrosine phosphorylation as previously demonstrated [McPartlin et al., 2011], and the presence of SPINKL also showed the same result (data not shown). Thus, we cannot conclude if SPINKL has any influence on Epac at this time. To resolve this, further studies are needed.

Chang [1957] reported that the decapacitation factor which exists in the seminal plasma can reverse capacitated sperm to an uncapacitated state. In this study, we found that the suppressive effect of SPINKL on sperm capacitation was invalid when sperm had

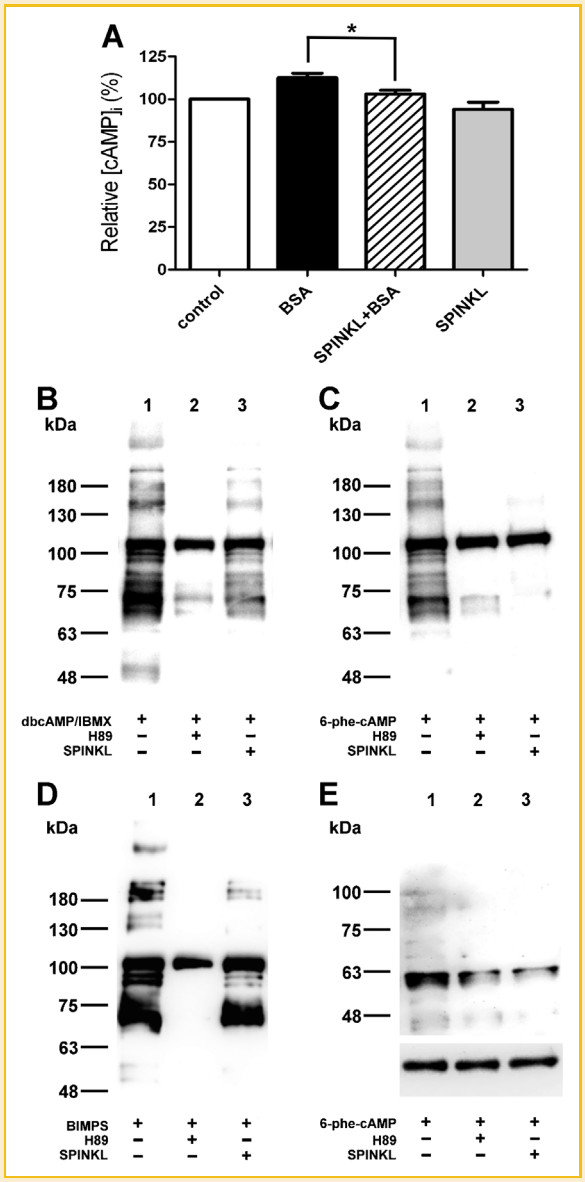


Fig. 7. SPINKL attenuated PKA-activated downstream sperm protein tyrosine phosphorylation. A: SPINKL suppressed BSA-induced intracellular cAMP elevation. Epididymal sperm cultured in HM medium were incubated with or without SPINKL (0.6 mg/ml) for 15 min, and then BSA was or was not added for an additional 90 min. After that, sperm lysates were extracted, and the concentration of cAMP was determined. Data are the mean ± SD of three independent experiments. Asterisks indicate significant differences compared with medium supplemented with BSA ($^*P < 0.05$). B–E: Effects of SPINKL on cAMP analog-induced sperm protein tyrosine phosphorylation. Epididymal sperm cultured in HM medium were incubated with H89 for 5 min or with SPINKL (0.6 mg/ml) for 15 min and then the cAMP analogs, dbcAMP (1 mM)/IBMX (100 μM) (B), 6-Phe-cAMP (1 mM) (C and E), or BIMPS (1 mM) (D), were added for an additional 90 min. Lane 1, without H89 or SPINKL but with the cAMP analog (positive control); lane 2, with H89 followed by the cAMP analog (negative control); lane 3, with SPINKL followed by the cAMP analog. The soluble fraction of the sperm lysate was resolved by SDS–PAGE, electrotransferred onto a nitrocellulose membrane, and immunoblotted with the anti-phosphotyrosine antibody (B–D), anti-phospho-SRC ($pTyr^{416}$) antibody (E), or anti-α-tubulin antibody (E).

previously been subjected to capacitation with capacitation-enhancing agents, suggesting that SPINKL cannot reverse sperm capacitation. We also provide in vitro and in vivo evidence demonstrating that SPINKL cannot reverse sperm capacitation. Thus, SPINKL is not a decapacitation factor. When sperm are not completely capacitated in vitro, SPINKL still had the ability to inhibit ongoing capacitation (Fig. 2, lane 5). But if sperm had been capacitated in vitro, SPINKL had no influence (Fig. 2, lanes 6 and 7). A similar situation was found for in vivo capacitated sperm (Fig. 3). Capacitated sperm would have lost their membrane cholesterol, thus leading to large amounts calcium influx. Adding SPINKL in that circumstance was too late to retrieve capacitation and the subsequent acrosome reaction.

SPINKL is a seminal vesicle-secreted glycoprotein [Lin et al., 2008]. It should be a component of the seminal plasma upon ejaculation. It was bound to sperm that exist in the uterus but had disappeared from sperm that exist in the oviduct during sperm transit through the female genital tract. Thus, SPINKL exerts its function in the uterus where the majority of sperm are uncapacitated. Murine sperm capacitation takes place in the oviduct where SPINKL no longer resides on the sperm surface. Therefore, SPINKL may serve as an uncapacitation factor in the uterus to prevent sperm from precocious capacitation and the subsequent acrosome reaction and thus preserve their fertilizing ability.

Cholesterol, sphingolipids, and associated proteins are all components of lipid rafts [Foster et al., 2003]. Some gangliosides and glycoproteins were demonstrated to have different distributions on the sperm surface before and after sperm capacitation [Shadan et al., 2004]. BSA and MBCD can induce cholesterol efflux from sperm plasma membranes, thus destroying the integrity of lipid rafts and promoting sperm capacitation [Choi and Toyoda, 1998; Cross, 1998; Visconti et al., 1999a1999b; Shadan et al., 2004]. Cholesterol efflux was demonstrated to mediate several events such as increases in the intracellular calcium ion concentration, increases in intracellular pH, migration of lipid raft components, and initiation of sperm protein tyrosine phosphorylation, which ultimately leads to capacitation and the subsequent acrosome reaction [Choi and Toyoda, 1998; Visconti et al., 1999a1999b; Shadan et al., 2004]. Nevertheless, the prior interaction of SPINKL with sperm can suppress the cholesterol efflux induced by BSA or MBCD and thus block capacitation signals. Glycodelin-S, a human seminal plasma protein, was also demonstrated to have the same effect on sperm; interestingly, it also has no capability to reverse human sperm capacitation and was suggested to be an uncapacitation factor [Chiu et al., 2005].

SPINKL may interact with components on lipid rafts to maintain the integrity of lipid rafts and thus prevent sperm capacitation. For example, SVS2, another mouse seminal vesicle-secreted protein, inhibits capacitation by binding to GM1 of lipid rafts to prevent sperm capacitation [Kawano et al., 2008]. ATP-binding cassette (ABC) transporter proteins were reported to be involved in the transport of cholesterol to cholesterol acceptors such as albumins, high-density lipoproteins, and apolipoproteins [Morales et al., 2008]. ABCA17 was found to be involved in the process of cholesterol efflux from mouse sperm plasma membranes [Morales et al., 2012]. Interestingly, it is present on the tail of mouse sperm,

the same binding site as SPINKL. In consideration that SPINKL blocks cholesterol efflux from sperm plasma membranes during capacitation and ABC transporter proteins are implicated in cholesterol transport, we suggest that the ABC transport protein on sperm might likely be the site where SPINKL exerts its action. However, this hypothesis needs to be further examined.

$Ca^{2+}$ influx into sperm is rapidly enhanced when calcium is supplemented to epididymal sperm being cultured in a calcium-free medium (our unpublished data). Rapid changes in intracellular ion concentrations are most probably elicited by ion channels since they are primarily responsible for rapid signaling events [Hille, 1992]. The effect of SPINKL on the change of $[Ca^{2+}]_i$ in freshly prepared epididymal sperm is to block extracellular $Ca^{2+}$ influx but not to enhance extrusion of intracellular $Ca^{2+}$ (our unpublished observations). How SPINKL blocks extracellular $Ca^{2+}$ influx is worth investigating, since this would help us better understand which sperm surface $Ca^{2+}$ channels are involved in extracellular $Ca^{2+}$ entry.

Several kinds of plasma membrane $Ca^{2+}$-permeable ion channels were found in sperm, including T-type voltage-gated $Ca^{2+}$ channels, transient receptor potential channels, cyclic nucleotide-gated channels, and CATSPER channels [Darszon et al., 2006]. BSA can induce extracellular $Ca^{2+}$ influx into sperm and thus lead to $[Ca^{2+}]_i$ increases [Xia and Ren, 2009]. Nevertheless, the $[Ca^{2+}]_i$ rise was prohibited by the application of SPINKL. Two phases of $[Ca^{2+}]_i$ rises were found for BSA-induced increases in $[Ca^{2+}]_i$ during capacitation [Xia and Ren, 2009]. The initial phase is dependent on the CASPER channel, while the second phase may be dependent on another yet to be determined channel. Since CATSPER channels are localized on a principal piece of mouse sperm and SPINKL is also bound at the tail of mouse sperm, SPINKL may act on the CATSPER or another $Ca^{2+}$-permeable ion channel.

In conclusion, SPINKL is able to block three early events in the sperm capacitation pathway that is cholesterol efflux from sperm plasma membranes, extracellular calcium ion influx into sperm, and intracellular cAMP increases, and downstream of cAMP/PKA signaling is probably mediated by attenuating SRC tyrosine kinase activity. Therefore, SPINKL is a potent inhibitor of sperm capacitation. However, SPINKL is not a decapacitation factor, since it cannot reverse sperm capacitation. These findings and SPINKL's binding to ejaculated sperm in the uterus but not in the oviduct suggest that SPINKL serves as a sperm protector in the uterus by preventing premature capacitation and thus preserves the fertilization potential before sperm reach the fertilization site.

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## REFERENCES

Austin CR. 1952. The capacitation of the mammalian sperm. Nature 170:326.

Baker MA, Hetherington L, Aitken RJ. 2006. Identification of SRC as a key PKA-stimulated tyrosine kinase involved in the capacitation-associated hyperactivation of murine spermatozoa. J Cell Sci 119:3182–3192.

Biggers JD, Whitten WK, Whittingham DG. 1971. The culture of mouse embryos in vitro. In: Daniels JC, editor. Methods in mammalian embryology. San Francisco, CA: Freeman Press. pp. 86–116.

Chang MC. 1951. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 168:697–698.

Chang MC. 1957. A detrimental effect of seminal plasma on the fertilizing capacity of sperm. Nature 179:258–259.

Chiu PC, Chung MK, Tsang HY, Koistinen R, Koistinen H, Seppala M, Lee KF, Yeung WS. 2005. Glycodelin-S in human seminal plasma reduces cholesterol efflux and inhibits capacitation of spermatozoa. J Biol Chem 280: 25580–25589.

Choi YH, Toyoda Y. 1998. Cyclodextrin removes cholesterol from mouse sperm and induces capacitation in a protein-free medium. Biol Reprod 59:1328–1333.

Cross NL. 1998. Role of cholesterol in sperm capacitation. Biol Reprod 59: 7–11.

Darszon A, Acevedo JJ, Galindo BE, Hernandez-Gonzalez EO, Nishigaki T, Trevino CL, Wood C, Beltran C. 2006. Sperm channel diversity and functional multiplicity. Reproduction 131:977–988.

de Jonge C. 2005. Biological basis for human capacitation. Hum Reprod Update 11:205–214.

de Lamirande E, Yoshida K, Yoshiike TM, Iwamoto T, Gagnon C. 2001. Semenogelin, the main protein of semen coagulum, inhibits human sperm capacitation by interfering with the superoxide anion generated during this process. J Androl 22:672–679.

de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL. 1998. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature 396:474–477.

Enserink JM, Christensen AE, de Rooij J, van Triest M, Schwede F, Genieser HG, Doskeland SO, Blank JL, Bos JL. 2002. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat Cell Biol 4:901–906.

Foster LJ, De Hoog CL, Mann M. 2003. Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. Proc Natl Acad Sci USA 100:5813–5818.

Fraser LR, Harrison RA, Herod JE. 1990. Characterization of a decapacitation factor associated with epididymal mouse spermatozoa. J Reprod Fertil 89: 135–148.

Go KJ, Wolf DP. 1985. Albumin-mediated changes in sperm sterol content during capacitation. Biol Reprod 32:145–153.

Hille B. 1992. Elementary properties of pores. In: Hille B, editor. Ionic channels of excitable membranes. Sunderland, MA: Sinauer Assoc. pp. 291–314.

Huang YH, Chu ST, Chen YH. 2000. A seminal vesicle autoantigen of mouse is able to suppress sperm capacitation-related events stimulated by serum albumin. Biol Reprod 63:1562–1566.

Hyne RV, Garbers DL. 1979. Regulation of guinea pig sperm adenylate cyclase by calcium. Biol Reprod 21:1135–1142.

Kawano N, Yoshida M. 2007. Semen-coagulating protein, SVS2, in mouse seminal plasma controls sperm fertility. Biol Reprod 76:353–361.

Kawano N, Yoshida K, Iwamoto T, Yoshida M. 2008. Ganglioside GM1 mediates decapacitation effects of SVS2 on murine spermatozoa. Biol Reprod 79:1153–1159.

Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM. 1998. A family of cAMP-binding proteins that directly activate Rap1. Science 282:2275–2279.

Lee MA, Storey BT. 1985. Evidence for plasma membrane impermeability to small ions in acrosome-intact mouse spermatozoa bound to mouse zonae pellucidae, using an aminoacridine fluorescent pH probe: time course of the zona-induced acrosome reaction monitored by both chlortetracycline and pH probe fluorescence. Biol Reprod 33:235–246.

Lee MA, Storey BT. 1986. Bicarbonate is essential for fertilization of mouse eggs: mouse sperm require it to undergo the acrosome reaction. Biol Reprod 34:349–356.

Lin MH, Lee RK, Hwu YM, Lu CH, Chu SL, Chen YJ, Chang WC, Li SH. 2008. SPINKL, a Kazal-type serine protease inhibitor-like protein purified from mouse seminal vesicle fluid, is able to inhibit sperm capacitation. Reproduction 136:559–571.

Lu CH, Lee RK, Hwu YM, Chu SL, Chen YJ, Chang WC, Lin SP, Li SH. 2011. SERPINE2, a serine protease inhibitor extensively expressed in adult male mouse reproductive tissues, may serve as a murine sperm decapacitation factor. Biol Reprod 84:514–525.

McPartlin LA, Visconti PE, Bedford-Guaus SJ. 2011. Guanine-nucleotide exchange factors (RAPGEF3/RAPGEF4) induce sperm membrane depolarization and acrosomal exocytosis in capacitated stallion sperm. Biol Reprod 85:179–188.

Morales CR, Marat AL, Ni X, Yu Y, Oko R, Smith BT, Argraves WS. 2008. ATP-binding cassette transporters ABCA1, ABCA7, and ABCG1 in mouse spermatozoa. Biochem Biophys Res Commun 376:472–477.

Morales CR, Ni X, Smith CE, Inagaki N, Hermo L. 2012. ABCA17 mediates sterol efflux from mouse spermatozoa plasma membranes. Histol Histopathol 27:317–328.

Naz RK, Rajesh PB. 2004. Role of tyrosine phosphorylation in sperm capacitation/acrosome reaction. Reprod Biol Endocrinol 2:75.

Ogreid D, Ekanger R, Suva RH, Miller JP, Doskeland SO. 1989. Comparison of the two classes of binding sites (A and B) of type I and type II cyclic-AMP-dependent protein kinases by using cyclic nucleotide analogs. Eur J Biochem 181:19–31.

Roberts KP, Wamstad JA, Ensrud KM, Hamilton DW. 2003. Inhibition of capacitation-associated tyrosine phosphorylation signaling in rat sperm by epididymal protein Crisp-1. Biol Reprod 69:572–581.

Sandberg M, Butt E, Nolte C, Fischer L, Halbrugge M, Beltman J, Jahnsen T, Genieser HG, Jastorff B, Walter U. 1991. Characterization of Sp-5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole-3′,5′-monophosphorothioate (Sp-5,6-DCl-cBiMPS) as a potent and specific activator of cyclic-AMP-dependent protein kinase in cell extracts and intact cells. Biochem J 279(Pt 2):521–527.

Shadan S, James PS, Howes EA, Jones R. 2004. Cholesterol efflux alters lipid raft stability and distribution during capacitation of boar spermatozoa. Biol Reprod 71:253–265.

Suarez SS. 2008. Regulation of sperm storage and movement in the mammalian oviduct. Int J Dev Biol 52:455–462.

Visconti PE, Kopf GS. 1998. Regulation of protein phosphorylation during sperm capacitation. Biol Reprod 59:1–6.

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS. 1995a. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development 121: 1129–1137.

Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P, Kopf GS. 1995b. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121:1139–1150.

Visconti PE, Galantino-Homer H, Ning X, Moore GD, Valenzuela JP, Jorgez CJ, Alvarez JG, Kopf GS. 1999a. Cholesterol efflux-mediated signal transduction in mammalian sperm. Beta-cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosine phosphorylation and capacitation. J Biol Chem 274:3235–3242.

Visconti PE, Ning X, Fornes MW, Alvarez JG, Stein P, Connors SA, Kopf GS. 1999b. Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. Dev Biol 214:429–443.

Ward CR, Storey BT. 1984. Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. Dev Biol 104:287–296.

Xia J, Ren D. 2009. The BSA-induced $Ca^{2+}$ influx during sperm capacitation is CATSPER channel-dependent. Reprod Biol Endocrinol 7:119.

Yanagimachi R. 1994. Mammalian fertilization. In: Knobil E, Neill JD, editors. The physiology of reproduction. New York, NY: Raven Press.