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Journal of Cellular Biochemistry 111:1188–1198 (2010)

Journal of Cellular Biochemistry

Capacitation Suppression by Mouse Seminal Vesicle Autoantigen Involves a Decrease in Plasma Membrane Ca^{2+}-ATPase (PMCA)-Mediated Intracellular Calcium

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ABSTRACT

Successful fertilization is tightly regulated by capacitation and decapacitation processes. Without appropriate decapacitation regulation, sperm would undergo a spontaneous acrosome reaction which leads to loss of fertilization ability. Seminal plasma is known to negatively regulate sperm capacitation. However, the suppressive mechanisms still remain unclear. In this study, we demonstrate the decapacitation mechanism of mouse seminal vesicle autoantigen (SVA) might target membrane sphingomyelin (SPM) and regulate plasma membrane Ca^{2+}-ATPase (PMCA) activity. The SVA was shown to suppress sperm capacitation induced by a broad panel of capacitation factors (bovine serum albumin (BSA), PAF, and cyclodextrin (CD)). Furthermore, SVA significantly decreased $[Ca^{2+}]_i$ and $NaHCO_3$-induced $[cAMP]_i$. Cyclic AMP agonists bypassed the SVA's suppressive ability. Importantly, the SVA may regulate PMCA activity which was evidenced by the fact that the SVA decreased the $[Ca^{2+}]_i$ and intracellular pH (pH_i) of sperm; meanwhile, a PMCA inhibitor (carboxyeosin) could reverse SVA's suppression of $[Ca^{2+}]_i$. The potential target of the SVA on membrane SPM/lipid rafts was highlighted by the high binding affinity of SPM–SVA (with a K_d of ~3 μM) which was close to the IC_{50} of SVA's suppressive activity. Additionally, treatment of mink lung epithelial cells with the SVA enhanced plasminogen activator inhibitor (PAI)-1 expression stimulated by tumor growth factor (TGF)-β and CD. These observations supported the membrane lipid-raft targeting of SVA. In summary, in this paper, we demonstrate that the decapacitation mechanism of the SVA might target membrane sphingolipid SPM and regulate PMCA activity to lower $[Ca^{2+}]_i$, thereby decreasing the $[cAMP]_i$ level and preventing sperm pre-capacitation. J. Cell. Biochem. 111: 1188–1198, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: DECAPACITATION; CALCIUM; PLASMA MEMBRANE Ca^{2+}-ATPase (PMCA); SEMINAL VESICLE; cAMP; SPERM; PROTEIN TYROSINE PHOSPHORYLATION

Sperm progressively acquire the ability to move but are still fertilization incompetent during epididymal transit. Sperm gain the fertilization capacity after residing in the female genital tract for a finite period of time. The physiological changes in sperm during this period are collectively called "capacitation." Capacitation is a complex process first described and defined independently by Chang [1951] and Austin [1952]. The capacitation process was demonstrated to involve alteration of the sperm plasma membrane, elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) and cAMP level ($[cAMP]_i$), increase of intracellular pH (pH_i), and stimulation of protein tyrosine phosphorylation. This leads to energy consumption, hypermotility, and eventually to an acrosome reaction of sperm [Visconti and Kopf, 1998].

The process of capacitation is tightly regulated by capacitation factors (such as serum albumin in the female reproductive tract) and suppression factors (in the epididymis and seminal vesicles).

Shing-Hwa Lu and Yuan-Kuei Yen contributed equally to this work.

Grant sponsor: National Science Council, Taiwan; Grant numbers: NSC98-3111-B-038-001, NSC99-2628-B-038-009-MY3; Grant sponsor: Taipei Medical University and Hospital; Grant number: 94TMU-TMUH-03.

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Received 19 May 2010; Accepted 4 August 2010 • DOI 10.1002/jcb.22844 • © 2010 Wiley-Liss, Inc.
Published online 17 August 2010 in Wiley Online Library (wileyonlinelibrary.com).

Inhibition of capacitation by suppression factors is referred to as "decapacitation" [Chang, 1957]. Inappropriate decapacitation regulation causes sperm to undergo a spontaneous acrosome reaction and lose the fertilization ability. The presence of decapacitation factors prevents sperm from undergoing pre-capacitation and allows effective fertilization at the right time and place. To date, several decapacitation factors were detected. Most of them are from the seminal plasma [Davis, 1974; Reyes et al., 1975; Eng and Oliphant, 1978; Begley and Quinn, 1982; Dostalova et al., 1994; Thérien et al., 1995; Lopes et al., 1998; Mortimer et al., 1998; Huang et al., 2000; Villemure et al., 2003; Zhu et al., 2006; Kawano et al., 2008]. For example, a sperm adhesion family of boar accessory sex gland fluids was reported to consist of decapacitation activity [Dostalova et al., 1994]; studies by Fraser [1998] suggested that the decapacitation mechanism involves fucose residues and a GPI-anchored receptor on epididymal sperm. Lopes et al. [1998] suggested that decapacitation activity was derived from manosyl glycopeptide of seminal plasma. The platelet-activating factor, acetylhydrolase, was also suggested to play a role in decapacitation by hydrolysis of PAF to lyso-PAF [Zhu et al., 2006]. In addition, the plasma membrane Ca^{2+}-ATPase (PMCA) was shown to be involved in decapacitation regulation [DasGupta et al., 1994; Adeoya-Osiguwa and Fraser, 1996]. Others are from the epididymis [Ni et al., 2009] or sperm-surface-associated proteins [Nixon et al., 2006]. However, the molecular mechanisms of these suppression factors in sperm decapacitation are not well defined.

Recently, we demonstrated that serum obtained from male and female mice immunized with seminal vesicle secretion fluid is immunoreactive to a seminal vesicle autoantigen (SVA) protein. The SVA is a 19-kDa androgen-responsive glycoprotein secreted from luminal epithelial cells of seminal vesicles [Huang et al., 1999]. The SVA is one of the major components in semen (~300 μM). By a thin-layer chromatography (TLC) overlay technique, SVA was shown to bind with choline-containing phospholipids such as sphingomyelin (SPM) and phosphatidylcholine (PC) [Huang et al., 1999]. Importantly, the SVA is able to suppress bovine serum albumin-(BSA) and platelet activating factor (PAF)-induced capacitation signals [Huang et al., 2000, 2005, 2007].

In this study, we further demonstrated the regulatory mechanism of the SVA in decreasing intracellular calcium ($[Ca^{2+}]_i$) which suppresses mouse sperm capacitation. We showed that the decapacitation mechanism of the SVA might interact with SPM/lipid rafts and stimulate PMCA to lower $[Ca^{2+}]_i$. The decrease in $[Ca^{2+}]_i$ would serve as the central effecter to decrease the $[cAMP]_i$, protein tyrosine phosphorylation, motility, and capacitation in mouse sperm.

MATERIALS AND METHODS

MATERIALS

Fatty acid-free bovine serum albumin (BSA), chlortetracycline (CTC), ionophore A23187, ruthenium red (RuR), 3-isobutyl-1-methylxanthine (IBMX), platelet-activating factor (PAF), SPM, 2-OH-β-cyclodextrin (2-OH-β-CD), polyvinylalcohol, N,N-dimethyl-formamide (DMF), and DMSO were from Sigma (St. Louis, MO); an anti-phosphotyrosine monoclonal antibody (clone 4G10) was from UBI (Lake Placid, NJ); a horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG) secondary antibody was from Jackson ImmunoResearch Lab (West Grove, PA); Percoll, chemi-luminescence detection ECL plus, and cAMP assay kits (RPN 255) were from Amersham Pharmacia Biotech (Buckinghamshire, UK); dibutylryl cAMP (dbcAMP) was from Research Biochemicals International (Natick, MA); Fluo-3-AM, BCECF-AM, and 5- (and 6) carboxyeosin diacetate (succinimidyl ester) (CE) were from Molecular Probes (Eugene, OR); and FITC-*Pisum sativum* agglutinin (FITC-PSA) was from Vector Lab (Burlingame, CA). All chemicals were of reagent grade.

PREPARATION OF SVA PROTEIN

The SVA protein used in this study was purified from seminal vesicles of outbred ICR mice as described previously [Huang et al., 1995]. In brief, the seminal vesicle secretion of ICR mice was dissolved in phosphate-buffered saline (PBS) buffer and stirred on ice for 1 h. The saline-dissolved protein extract was purified by CM-Sephadex C-25 chromatography, followed by gel filtration with a Sephadex G-75 column (2.5 cm × 75 cm) and a protein-pak DEAE 5 pw column (7.5 cm × 75 mm, 10 μm) equilibrated with 0.01 M ammonium acetate (pH 6.8). The DEAE column was eluted with a linear gradient of 0–60% ammonium acetate at a flow rate of 1.0 ml/min for 25 min. The purity of the SVA protein was detected by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) in conjunction of a Western blot analysis (data not shown). The concentration of the SVA was determined by a BCA protein quantization assay kit from Pierce (Rockford, IL).

PREPARATION OF SPERM

Outbred CD-1 mice purchased from Charles River Laboratories (Wilmington, MA) were bred in the Animal Center at the School of Medicine, Taipei Medical University. Animals were handled in accordance with institutional guidelines on animal experimentation.

The culture medium used throughout these studies was modified Krebs Ringer bicarbonate HEPES medium (HM) as described previously [Huang et al., 2000]. The pH of the medium was adjusted to 7.3–7.4 with humidified air/CO_2 (95:5) in an incubator at 37°C for 48 h before use. Polyvinylalcohol (1 mg/ml) was added to serve as a sperm protectant. Mature mouse sperm with good motility were harvested by a swim-up procedure from the caudal epididymides. The viability and progressive motility of the sperm fraction used in the present study were both >90%.

CYTOLOGICAL OBSERVATIONS

The population of mouse sperm at the capacitated stage was analyzed by the CTC method [Ward and Storey, 1984], and the ionophore A23187-induced acrosome reaction was determined by the FITC-PSA staining method [Centola et al., 1990]. The A23187 ionophore was first dissolved in DMF and diluted with modified HM. After mouse sperm were treated with BSA or various reagents, 5 μM of the A23187 ionophore was added for an additional 30 min to induce the acrosome reaction. The sperm suspension was smeared on a slide and air dried, followed by fixing in methanol. The sperm smear was stained with FITC-PSA (1:50 dilution in PBS) for 10 min.

Sperm with green fluorescence over the acrosome region were defined as acrosome-intact, while the ones without green fluorescence bands were defined as acrosome-reacted. The percentage of acrosome-reacted sperm was obtained by counting 200 sperm in each smear, and the mean was derived from averaging the counts in at least three separate experiments.

DETECTION OF PROTEIN TYROSINE PHOSPHORYLATION

Sperm (5×10^6 cells/ml) were incubated under different experimental conditions (such as 0.3% BSA, 3 mM 2-OH-β-CD, and 5 μM PAF) in the presence or absence of the SVA (66 μM). In some experiments, dbcAMP (a cAMP agonist, 1 mM) and IBMX (a phosphodiesterase inhibitor, 100 μM) were added at 37°C for 90 min. The cell lysate was prepared according to Visconti et al. [1995a], subjected to a 10% SDS–PAGE, and then transferred to a PVDF membrane for Western blot analysis. The monoclonal anti-phosphotyrosine IgG (clone 4G10, 1 μg/ml) was used as the primary antibody and HRP-conjugated anti-mouse IgG (1:2,000) served as the secondary antibody. The enzyme activity of HRP was detected by the ECL system according to the manufacturer's instructions.

MEASUREMENT OF THE cAMP CONTENT

Cauda epididymal spermatozoa (1×10^6 cells) were incubated with different concentrations of the SVA (0-1 μM) for 15 min at 37°C in HCO_3^--containing HM medium. Treatment was terminated by adding 1 ml 0.1% TCA in 95% ethanol, and cAMP was extracted. The accumulative amount of cAMP in sperm was determined using a competitive nonacetylation immunoassay kit (RPN 255, Amersham Pharmacia Biotech), according to the manufacturer's instructions.

ASSAY OF SPERM MOTILITY BY A COMPUTER-ASSISTED SPERM ASSAY (CASA)

The percentage of sperm motility was determined by a CASA with a sperm motility analyzer (IVOS version 10; Hamilton-Thorne Research, Beverly, MA). Parameters of the CASA analyzer and experimental procedure were as described in a previous report [Huang et al., 1999].

DETERMINATION OF INTRACELLULAR CALCIUM LEVEL AND PH VALUE

5-(and 6) Carboxyeosin diacetate (succinimidyl ester) (CE) was dissolved in DMSO to 10 mM as a stock solution. To block Ca^{2+} efflux, sperm were loaded with 0–50 μM CE for 10 min. Cells were then incubated with a carboxyeosin-free solution for a further 15 min to allow for carboxyeosin de-esterification.

Sperm $[Ca^{2+}]_i$ was determined using fluo-3 AM by flow cytometry (FACScan, BD). In brief, mouse sperm were loaded with fluo-3 AM (10 μM) for 10 min, then sperm cells were washed twice with calcium-free modified HM to remove any free fluo-3 AM. Fluo-3 AM-loaded cells (10^6 cells/ml) were treated with different concentrations of the SVA (0–66 μM) and analyzed by flow cytometry.

The intracellular pH value (pH_i) was determined using BCECF-AM. In brief, mouse sperm were loaded with BCECF-AM (2 μM) for 10 min, then cells were washed twice with modified HM to remove any free fluore. Fluore-loaded sperm were treated with the SVA (0–66 μM) and then analyzed by flow cytometry. For pH_i calibration, a nigericin/high-K^+ calibration protocol was used to derive the pH_i values as described previously [Zeng et al., 1996; Huang et al., 2005]. The fluorescence of fluo-3 was excited at 488 nm and measured via a 515- to 540-nm filter, and the fluorescence of BCECF was excited at 510 nm and measured via a 564- to 606-nm filter. PMT voltages and gains were set to optimize the dynamic range of the signal. The fluorescence intensity of sperm was quantified for 10,000 individual sperm cells.

FLUORESCENCE SPECTRA

The fluorescence intensity of the SVA in Tris-buffered saline (TBS) at pH 7.4 was measured and expressed in arbitrary units using a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan). Both the excitation and emission slit-widths were 10 nm. Raman emission due to the scattering of the solvent was minimized by adjusting the intensity scale. No more than 5 min was used to scan a spectrum to avoid protein denaturation. The fluorescence intensity at wavelength λ_2 (nm) while the fluorophore was excited at wavelength λ_1 (nm) is denoted by $F_{\lambda_1}^{\lambda_2}$. A modified Scatchard plot [Huang et al., 1995] was constructed to analyze the fluorescence data of a complex formed by the SVA with SPM:

$$\frac{|\Delta F|}{[L]_{free}} = \frac{\Delta F_\infty}{K_d} - \frac{|\Delta F|}{K_d} \quad (1)$$

where ΔF is the change in the protein fluorescence with the addition of the ligand, L, F_∞ is the protein fluorescence in the absence of the ligand, and K_d is the dissociation constant of the complex. Throughout the titration, $|\Delta F|/[L]_{total}$ was plotted against $|\Delta F|$, because $[L]_{free}$ was close to $[L]_{total}$.

FLUORESCENCE LOCALIZATION OF THE GM1 DISTRIBUTION IN MOUSE SPERM

Localization of GM1 was visualized with cholera toxin subunit B (CTB). Mouse sperm cells under different capacitation conditions, such as the control group (uncapacitated sperm), BSA (0.3%) (capacitated sperm), and BSA (0.3%) + SVA (20 μM) (decapacitated group), were fixed for 10 min while in suspension by adding an equal volume of 0.008% paraformaldehyde in phosphate-buffered saline (PBS). Sperm were then washed with PBS and incubated for 10 min with CTB (5 μg/ml). Sperm were washed again, divided into aliquots, placed directly on slides for microscopy, and then mounted using a GVA mountant (Invitrogen).

STATISTICAL ANALYSIS

All experiments were repeated at least three times with three different pooled sperm samples from four or five male mice. Data are expressed as the mean ± SD. Differences in means were assessed by a *t*-test or one-way analysis of variance (ANOVA) and post-hoc test (GraphPad Instat3 program).

RESULTS

SUPPRESSIVE EFFECT OF SVA ON MOUSE SPERM CAPACITATION

The SVA is secreted from seminal vesicles and is the major component of semen [Huang et al., 1999]. We previously demonstrated the suppressive effect of the SVA on mouse sperm

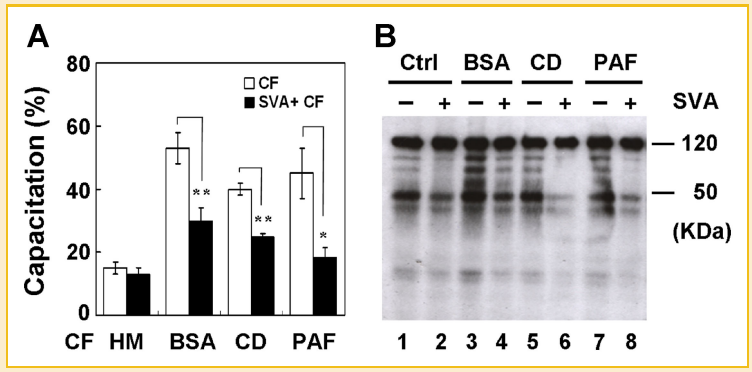

Fig. 1. Suppressive effect of the SVA on mouse sperm capacitation and protein tyrosine phosphorylation. A: Mouse sperm were incubated under different experimental conditions to induce sperm capacitation. HM medium alone served as a control of "CF" group; and (SVA + HM medium) was the control of the "SVA + CF" group. The concentrations of the agents were 0.3% for BSA, 3 mM 2-OH-β-CD, 5 μM PAF, and 66 μM SVA. The CTC fluorescence method was used to define capacitated sperm. The percentages of capacitated sperm under different experimental conditions in the absence (open columns) or presence of SVA (closed columns) are shown. The mean ± SD of three independent determinations for each condition is shown. *Indicates a significant difference from samples under different experimental conditions (as indicated) at $P < 0.05$ and ** at $P < 0.01$ with *t*-test. B: The protein tyrosine phosphorylation of sperm under different experimental conditions in (A) is shown. CF, capacitation factor.

capacitation [Huang et al., 2000]. To further confirm the SVA's suppressive ability, we treated sperm with the SVA supplemented with well-defined putative capacitation factors, such as BSA, cyclodextrin (CD, 2-OH-β-CD), and PAF. The capacitation states of mouse caudal epididymal sperm were determined by a CTC assay. HM medium alone served as a control of "CF" group; and (SVA + HM medium) was the control of "SVA + CF" group. As shown in control panel of Figure 1A, <15% of sperm underwent capacitation under control condition (HM medium alone, open column) or SVA (66 μM) treatment (closed column). BSA (0.3%), CD (3 mM), and PAF (5 μM) significantly induced mouse sperm capacitation (by 40–55%, open columns of the BSA, CD, and PAF panels). Supplementation with the SVA (66 μM) in the HM medium significantly suppressed BSA-/CD- and PAF-induced sperm capacitation (Fig. 1, closed vs. open columns). The suppressive effect of the SVA on mouse sperm capacitation was further confirmed by examination of capacitation-associated protein tyrosine phosphorylation of sperm [Visconti et al., 1995a,b; Visconti and Kopf, 1998]. As shown in Figure 1B, the capacitation-associated protein tyrosine phosphorylation of sperm induced by capacitation factors was significantly suppressed by the SVA's action (Fig. 1B, lane 4 vs. 3; lane 6 vs. 5; lane 8 vs. 7).

BYPASSING SVA'S SUPPRESSIVE ACTIVITY ON MOUSE SPERM CAPACITATION BY cAMP ANALOGS

cAMP was demonstrated to induce sperm capacitation and its associated protein tyrosine phosphorylation [Visconti et al., 1995b]. Given that the SVA exhibited the ability to suppress capacitation and its associated protein tyrosine phosphorylation in sperm, we then examined the active role of cAMP in SVA's suppressive activity. As shown in Figure 2, adding dbcAMP (a cAMP agonist)/IBMX (a cyclic nucleotide phosphodiesterase) bypassed the SVA's suppressive effect on mouse sperm capacitation (Fig. 2A) as well as its associated protein tyrosine phosphorylation (Fig. 2B). These observations suggested upstream regulator of SVA on cAMP-mediated intracellular signals in mouse sperm capacitation.

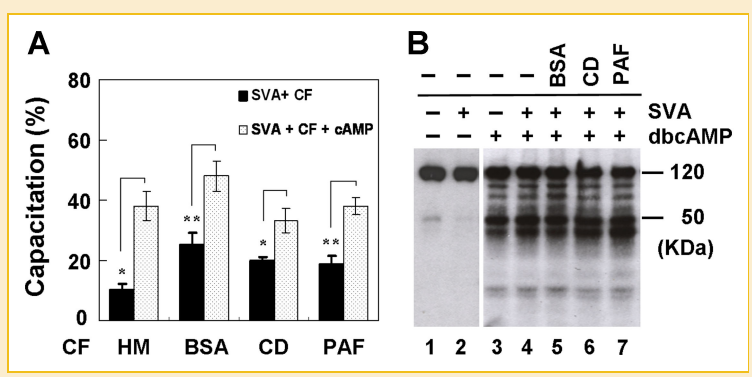

Fig. 2. Bypassing the SVA's suppressive activity of mouse sperm by cAMP analogs. A: Mouse sperm were incubated under different experimental conditions. The concentrations of the agents were 0.3% for BSA, 3 mM for 2-OH-β-CD, 5 μM for PAF, and 66 μM for the SVA. The percentages of capacitated sperm under different SVA suppression conditions in the absence (closed columns) or presence of cAMP agonist dbcAMP (1 mM)/IBMX (100 μM) (open columns) are shown. The mean ± SD of three independent determinations for each condition is shown. *Indicates a significant difference from samples under different experimental conditions (as indicated) at $P < 0.05$ and ** at $P < 0.01$ with *t*-test. B: Protein tyrosine phosphorylation of sperm under different experimental conditions in (A) is shown.

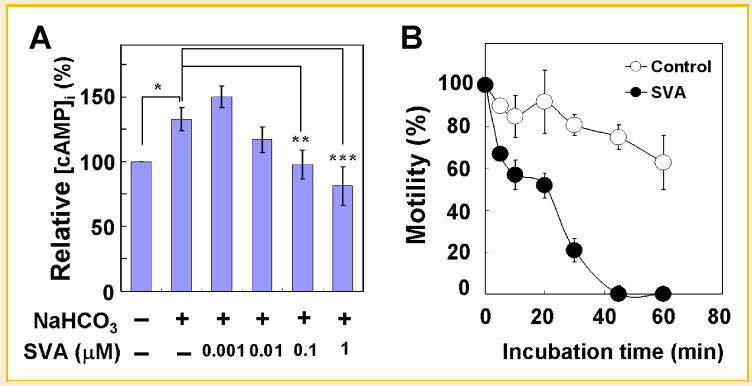

Fig. 3. Suppressive effect of the SVA on motility and $NaHCO_3$-induced $[cAMP]_i$ of mouse sperm. A: $[cAMP]_i$ levels of sperm under HCO_3^- treatment in Ca^{2+}-containing HM are shown. The relative $[cAMP]_i$ levels in sperm cells without HCO_3^- or SVA treatment were set to 100%. The $[cAMP]_i$ levels of sperm treated with Ca^{2+} (1.7 mM)/HCO_3^- (40 mM)-containing HM served as the positive control. The relative $[cAMP]_i$ levels in sperm cells treated with increasing concentrations of the SVA (0–1 μM) in Ca^{2+}/HCO_3^--containing HM are shown. The mean ± SD of five independent determinations for each condition is shown. B: Mouse sperm in modified HEPES medium (HM) at pH 7.4 (open circles) or in the presence of the SVA (66 μM) (closed circles) were incubated under 5% CO_2 at 37°C for 0–60 min. The relative percentage of sperm motility was measured by a computer-assisted sperm assay (CASA) as described in the text. Results of the determination at each specified condition are expressed as percentages of control cell motility measured at time zero incubation (closed vs. open circles). Points are the mean ± SD of 10 determinations. * Indicates a significant difference from samples without SVA exposure (0 μM) at $P < 0.05$, ** at $P < 0.01$, and *** at $P < 0.001$ with one-way ANOVA and post hoc test. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

SUPPRESSIVE EFFECT OF SVA ON THE MOTILITY AND $NaHCO_3$-INDUCED INTRACELLULAR cAMP LEVEL IN SPERM

In our previous reports, we demonstrated the suppressive ability of the SVA protein on the intracellular cAMP levels ($[cAMP]_i$) in mouse sperm [Huang et al., 2005, 2007]. SVA apparently acts upstream in regulating cAMP-mediated signals as the cAMP agonists are able to bypass the SVA suppression ability. Soluble adenylyl cyclase (sAC) is an essential component of cAMP-signaling cascades that activate sperm motility and capacitation; and, is known to be dominantly regulated by HCO_3^- and $[Ca^{2+}]_i$ [Hess et al., 2005]. To examine whether SVA affects sAC activity, we then tested SVA's effect on $[Ca^{2+}]_i$ and $NaHCO_3^-$-induced $[cAMP]_i$ in sperm. As shown in Figure 3A, HCO_3^- was used as a stimulant to significantly elevate $[cAMP]_i$ (a 40% increase, $P < 0.001$) [Litvin et al., 2003]. The SVA significantly suppressed HCO_3^--induced sperm $[cAMP]_i$ in a dose-dependent manner (0–1 μM) (Fig. 3A). The $[cAMP]_i$ of sperm which cultured in HCO_3^--free HM served as a basal control (set to 100%).

Given sAC-mediated $[cAMP]_i$ level affects sperm motility [Hess et al., 2005], we then examined the effect of SVA on mouse sperm motility by a CASA analysis. As shown in Figure 3B, most of the spermatozoa freshly retrieved from a mouse caudal epididymis retained their motility even after incubation in modified HM at 37°C for 120 min [Huang et al., 1999]. Adding the SVA (66 μM) to the medium significantly decreased the sperm motility. The suppressive effect of SVA on sperm motility was time dependent when compared to the control group (Fig. 3B, closed vs. open circles). After 40 min of incubation, sperm motility was completely suppressed in the presence of SVA (Fig. 3B, closed circles).

THE SVA DECREASED $[Ca^{2+}]_i$ THROUGH REGULATING PMCA

Ca^{2+} is known to act upstream of BSA-induced sperm capacitation [Visconti and Kopf, 1998]. Our previous reports demonstrated the suppressive effect of the SVA on both BSA- and PAF-induced $[Ca^{2+}]_i$ in mouse sperm [Huang et al., 2005, 2007]. The decrease in $[Ca^{2+}]_i$ of sperm apparently plays a critical role in the SVA's suppressive activity. In cells, $[Ca^{2+}]_i$ decreasing may be due to blocking of extracellular Ca^{2+} influx and/or enhancing of intracellular Ca^{2+} clearance. To determine how the SVA suppresses $[Ca^{2+}]_i$, we first tested the SVA's effect on Ca^{2+} influx of sperm. To determine this, sperm were incubated with increasing concentrations of the SVA in an EDTA-containing Ca^{2+}-free medium. As shown in Figure 4A, the SVA significantly and dose-dependently decreased the $[Ca^{2+}]_i$ of sperm in EDTA-supplemented Ca^{2+}-free medium. This observation strongly implies that the decrease in $[Ca^{2+}]_i$ due to SVA's suppression is not through blocking of extracellular Ca^{2+} influx from the plasma membrane.

We then examined the effect of the SVA on intracellular calcium clearance. Four major Ca^{2+} clearance mechanisms are well documented in most cell types. Two of them are on plasma membrane, for example, plasma membrane Ca^{2+}–ATPase (PMCA) and the Na^{2+}–Ca^{2+} exchanger (NCX); others are in intracellular organelles, for example, sarcoplasmic endoplasmic reticulum Ca^{2+}–ATPase (SERCA pumps) and the mitochondrial Ca^{2+} uniporter (MCU) [Wennemuth et al., 2003]. SERCA pumps are not dominant in $[Ca^{2+}]_i$ clearance in mouse sperm [Wennemuth et al., 2003]. In our experiments, adding the MCU-specific inhibitor (RuR) to the reaction medium failed to recover the suppressive effect of the SVA on $[Ca^{2+}]_i$ (data not shown). These observations rule out the possibility of an intracellular organelle clearance mechanism, and further hint at the possibility of PMCA and NCX being involved in SVA's suppressive activity.

PMCA is known to act by exporting cytoplasmic Ca^{2+} along with importing one to two extracellular protons. This transport system leads to decreases in $[Ca^{2+}]_i$ and the intracellular pH value (pH_i) in cells. Under decapacitation culture conditions, we found that the SVA dose-dependently decreased both the basal $[Ca^{2+}]_i$ and pH_i (Fig. 4). This observation hinted at the potential of PMCA to target the SVA. To test this hypothesis, fluo-3 AM was used as the $[Ca^{2+}]_i$

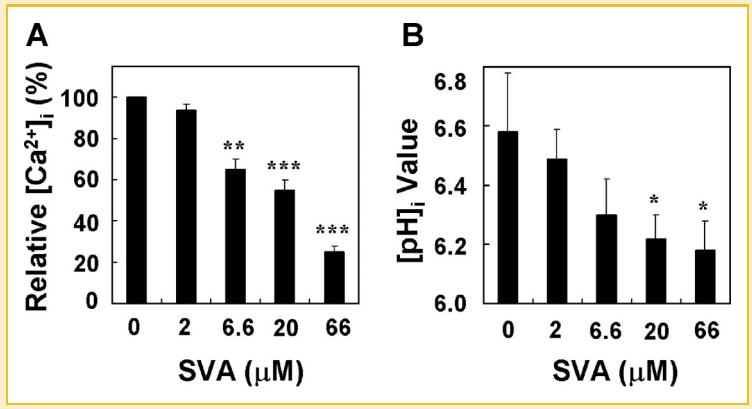

Fig. 4. Suppressive effect of the SVA on endogenous $[Ca^{2+}]_i$ and pH_i in mouse sperm. A: Sperm pre-loaded with fluo-3 AM in EDTA-containing Ca^{2+}-free medium, or (B) with BCECF-AM in Ca^{2+}-containing medium were incubated with increasing concentrations of the SVA (0–66 μM). After 60 min, sperm were collected, and the fluorescence intensity was analyzed by flow cytometry. The relative $[Ca^{2+}]_i$ is shown with $[Ca^{2+}]_i$ in sperm samples without SVA treatment designated as 100% (A). For the pH_i value, the fluorescence intensity of sperm treated with or without the SVA was determined and normalized to a pH 6–7 standard curve. The mean ± SD of more than three independent determinations for each condition is shown. ** Indicates a significant difference from samples without SVA treatment at $P < 0.01$ and *** at $P < 0.001$ (A). * Indicates a significant difference from samples without SVA exposure at $P < 0.05$ (B) with one-way ANOVA and post hoc test.

tracer. Sperm pre-loaded with fluo-3-AM (10 μM) were incubated with the SVA (66 μM) in the presence or absence of carboxyeosin (CE, an inhibitor of PMCA) [Gatto and Milanick, 1993]. As shown in Figure 5A, the SVA significantly decreased the $[Ca^{2+}]_i$-derived fluorescence intensity of sperm cells (panel b vs. a, epifluorescence microscopic analysis, indicated by green color). The suppressive effect of the SVA on $[Ca^{2+}]_i$ was effectively reversed by the addition of CE at 0.125 μM (panel c vs. panel a). The reversal effect of CE on SVA suppression was dose-dependent (Fig. 5B). In the absence of CE, the SVA (66 μM) caused a 40% decrease in the $[Ca^{2+}]_i$ of sperm (closed circles at [CE] = 0). Adding CE to the reaction medium significantly reversed the SVA's suppression of $[Ca^{2+}]_i$ of sperm. At 5 μM, CE restored the SVA's suppression of $[Ca^{2+}]_i$ from 40% to 20% (with a 50% recovery rate). At 20–50 μM, CE almost completely reversed the SVA's suppressive effect (Fig. 5B). In addition, CE (0.125 μM) also reversed the SVA's suppression of the A23187-induced acrosome reaction in mouse sperm (Fig. 5D). Together, these results strongly support the involvement of PMCA in the SVA's suppression of $[Ca^{2+}]_i$ in mouse sperm.

BINDING OF THE SVA TO SPHINGOMYELIN (SPM)

We previously demonstrated the binding of SPM to the SVA [Huang et al., 1999]. Herein, we further determined the binding affinity of SPM–SVA by a tryptophan fluorescence analysis. There are two tryptophan residues, Trp^2 and Trp^{82}, located on the SVA protein surface which results in an emission peak at 345 nm when excites the SVA protein/TBS at 295 nm [Huang et al., 1995] (Fig. 6, solid line). The presence of 5.0% (v/v) ethanol in the protein solution did not change either the spectral profile or emission intensity of the protein fluorescence (solid vs. dotted line), indicating the lack of a solvent effect on tryptophan's status. In contrast, the addition of 40 μM SPM (in 0.5% (v/v) ethanol) in the protein solution considerably enhanced the protein fluorescence intensity but did not shift the emission peak (dotted-dashed vs. solid line). Apparently, SPM interacted with the SVA to change the protein's fluorescence. Therefore, SPM–SVA binding was probed by the ability of SPM to perturb the protein fluorescence. We fitted the $F^{\lambda 2}_{\lambda 1}$ values, obtained by titrating 1.6 μM of the protein solution with SPM, to Equation (1). As shown in the inset of Figure 6, the modified Scatchard plot was linear, supporting there being a single type of SPM binding site on the SVA protein. The apparent K_d of SPM–SVA binding was estimated to be $(1.89 \pm 0.02) \times 10^{-6}\,M^{-1}$, which is compatible with the IC_{50} of the SVA's suppression of sperm capacitation and protein tyrosine phosphorylation.

Given SPM is one of the major components of membrane lipid raft, we examined the SVA targeting on the membrane lipid raft. Mink lung epithelial cells (MvlLu) and TGF-β system were used in this experiment. TGF-β has been demonstrated to target on the membrane lipid raft to stimulate the expression of plasminogen activator inhibitor-1 (PAI-1) [Chen et al., 2008]. In this aspect, we then test the SVA effect on TGF-β-induced PAI-1 expression of MvlLu cells. As shown in Table I, further treating MvlLu cells with the SVA enhanced the PAI-1 expression stimulated by TGF-β and CD (Table I). TGF-β significantly increased PAI-1 expression compared to the control group (10-fold for exp. group 2). CD showed to modulate Cholesterol in lipid rafts thereby to enhance cellular TGF-β responsiveness of PAI-1 (exp. group 4 vs. 2, from 10- to 63-fold). In our experiments, the SVA consistently increased the TGF-β responsive PAI-1 expression by 2-fold (exp. group 2 vs. 6, from 10- to 22-fold (TGF-β group); and group 4 vs. 7, from 63- to 112-fold (TGF-β plus CD group)). This observation suggests that the target of the SVA in decapacitation regulation is on lipid rafts.

THE SVA DID NOT AFFECT THE BSA-INDUCED LIPID-RAFT DISTRIBUTION OF MOUSE SPERM

Given that the SVA binds SPM and targets PMCA, we then examined whether the SVA alters the BSA-induced lipid-raft distribution of sperm. For this purpose, a GM1 antibody was used to probe the caveolae/raft domain in sperm, and the entire sperm population was

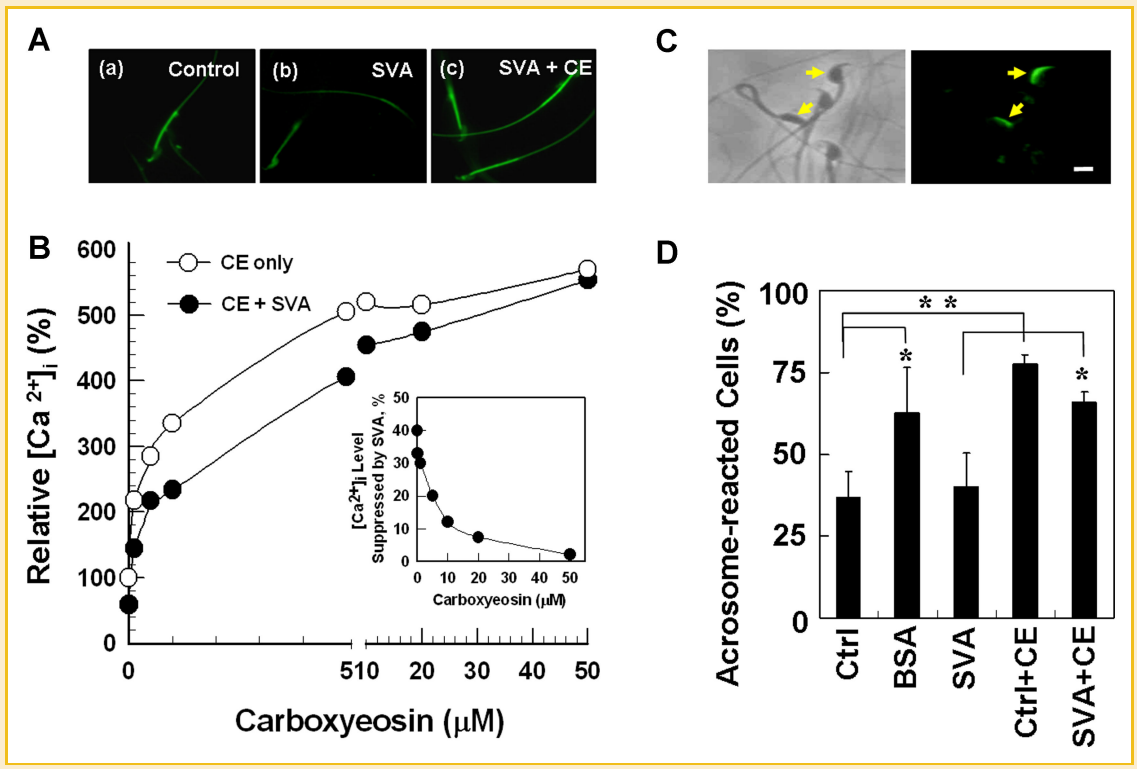

Fig. 5. Carboxyeosin-AM (CE) reversal of the SVA suppression of $[Ca^{2+}]_i$ and the ionophore A23187-induced acrosome reaction in mouse sperm. Mouse sperm pre-loaded with fluo-3 AM and CE (0–50 μM) were incubated with or without the SVA (6.6 μM). After 30 min of incubation, sperm were collected, and the fluorescence intensity was analyzed by flow cytometry and expressed as a percentage of sperm without SVA treatment. A: Representative images showing the fluorescence intensity in sperm under different conditions reflecting corresponding changes in $[Ca^{2+}]_i$: control (HM only, panel a), SVA (panel b), and SVA + CE (0.125 μM) (panel c). B: The $[Ca^{2+}]_i$ level of sperm suppressed by the SVA under CE treatment is shown. The relative $[Ca^{2+}]_i$ in sperm treated with CE or CE + SVA are shown in the inset of (B). The fluorescence intensity of sperm not treated with the SVA or CE was set to 100%. C: Acrosome-reacted sperm were detected using the FITC-*Pisum sativum* agglutinin (FITC-PSA) fluorescence method. The intact acrosome cap is indicated by an arrow. D: The number of A23187-induced acrosome-reacted sperm was estimated and is expressed as the percentage of the control. The mean ± SD of three independent determinations for each condition is shown. * Indicates a significant difference from a specific experimental condition (as indicated) at $P < 0.05$ and ** at $P < 0.01$. Bar = 10 μM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

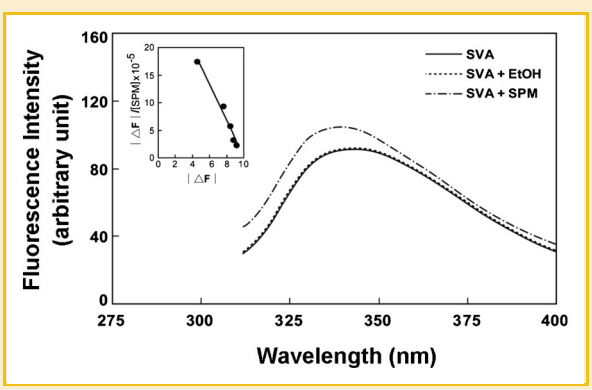

Fig. 6. Analysis of the interaction of the SVA with SPM using tryptophan fluorescence spectrometry. Emission spectra of the SVA (1.6 μM) with or without SPM (40 μM) were scanned at an excitation wavelength of 295 nm. The emission spectra of the SVA protein in TBS is expressed as the solid line; 5% ethanol (v/v) in the SVA protein solution is shown as the dotted line, and SPM in 1% ethanol (v/v) plus the SVA protein is shown as the dotted-dashed line. The modified Scatchard plot for the binding SPM to the SVA is given in the inset. The correlation coefficient was calculated to be >0.98.

identified as having an acrosomal plasma membrane (APM), diffuse pattern (D), apical acrosome/post-acrosomal (AA/PA), post-acrosomal plasma membrane (PAPM), and acrosome (AR) (Fig. 7A) [Buttke et al., 2006; Selvaraj et al., 2007]. In the control group (uncapacitated sperm), the GM1 distribution in sperm was mostly located at AA/PA (Fig. 7B, white column). In the BSA group (capacitated sperm), the major GM1 distribution of sperm had

TABLE I. Effect of SVA on PAI-1 Expression Stimulated by TGF-β and CD

Exp group	Treatment	PAI-1 expression (fold)
1	Control	1
2	TGF-β (20 pM)	10
3	CD (10 mg/ml)	2
4	TGF-β (20 pM) + CD (10 mg/ml)	63
5	SVA (20 μM)	3
6	SVA (20 μM) + TGF-β (20 pM)	22
7	SVA (20 μM) + TGF-β (20 pM) + CD (10 mg/ml)	112

[a]MvlLu cells were treated with CD, SVA, or CD + SVA at 37°C for 1 h. After incubation of cells with and without TGF-β for 2 h, the expression of PAI-1 was then determined by real time RT-PCR.

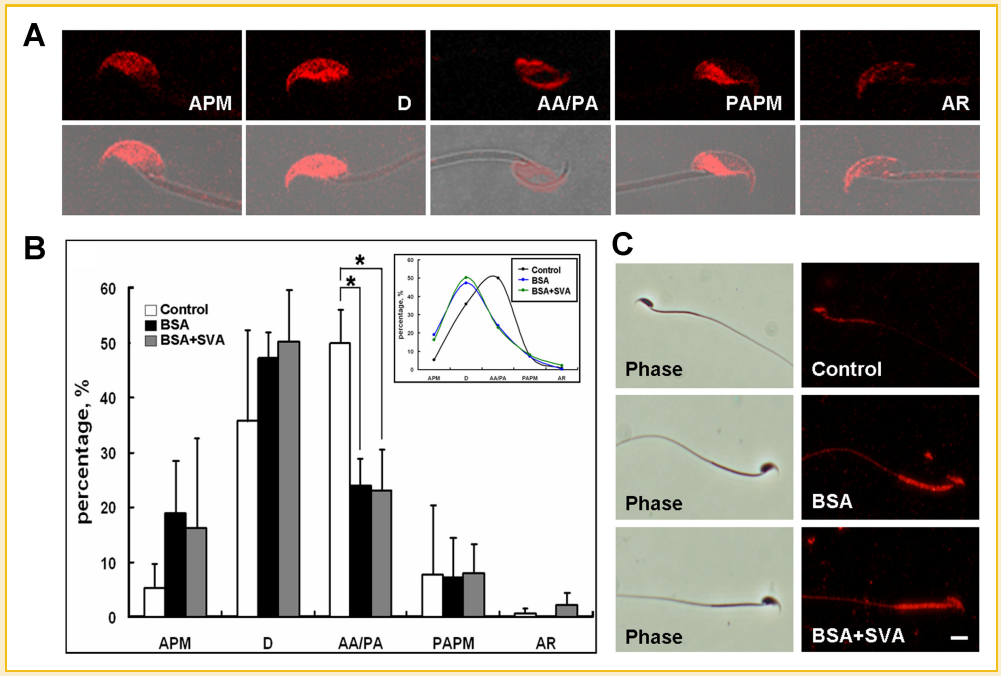

Fig. 7. Non-lipid raft re-distribution of SVA suppression of mouse sperm. A: GM1 distribution pattern in sperm was well identified as APM, D, AA/PA, PAPM, and AR. B: Percentages of the GM1 distribution pattern of control sperm (uncapacitated), BSA group (capacitated), and BSA + SVA group (decapacitated) are shown. C: The caveolin distribution pattern of control sperm (uncapacitated, white column), BSA group (capacitated, black column), and BAS + SVA group (decapacitated, gray column) are shown. * Indicates a significant difference from a specific experimental condition at $P < 0.05$. Bar = 10 μM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

shifted from AA/PA to the D pattern (Fig. 7B, black column). In our experiments, SVA did not affect the BSA-induced GM1 distribution from D back to AA/PA (Fig. 7B, gray column and inset figure). This observation was further confirmed by probing with the caveolin protein which is enriched in lipid-raft domains (Fig. 7C), suggesting that the regulation of SVA on PMCA activity is not through re-distribution of lipid-raft components.

DISCUSSION

Functional fertilization is regulated by the appropriate processes of capacitation (activation) and decapacitation (suppression). Capacitation is the prerequisite process in which sperm gain the ability for successful fertilization. But, without suppressive regulation, sperm may undergo a spontaneous acrosome reaction and lose their fertilization ability before they meet an oocyte at the oviduct. Seminal plasma contains high concentrations of suppression factors (decapacitation factors) to prevent the unfruitful capacitation and spontaneous acrosome reaction. However, the suppressive mechanisms of seminal plasma proteins are not well defined.

In in vitro culture systems, Ca^{2+}, HCO_3^-, and cholesterol acceptors such as BSA [Visconti et al., 1995a] and β-cyclodextrin [Visconti et al., 1999] are essential components for supporting sperm capacitation. Regulation of the intracellular Ca^{2+} concentration plays a critical role in sperm activation. Our previous studies demonstrated the SVA's suppressive ability of mouse sperm capacitation [Huang et al., 2000, 2005, 2007]. In this paper, we further demonstrate that the SVA-mediated sperm decapacitation involves a decrease in $[Ca^{2+}]_i$ regulated by PMCA. The SVA significantly decreased both the basal $[Ca^{2+}]_i$ and cAMP levels, sperm motility, and the putative capacitation factors (BSA, 2-OH-β-CD, and PAF)-induced capacitation and its associated protein tyrosine phosphorylation (Figs. 1–4). Importantly, the SVA significantly suppressed the HCO_3^--induced $[cAMP]_i$ of sperm (Fig. 3). HCO_3^- and Ca^{2+} are known to activate a soluble adenylyl cyclase sAC. The activated sAC then regulates intracellular cAMP signaling thereby activating sperm motility and enables sperm to penetrate the oocyte zona pellucida [Chen et al., 2000; Jaiswal and Conti, 2003; Litvin et al., 2003]. In our experiments, the decrease in $[Ca^{2+}]_i$ appeared to play an important role in the SVA's decapacitation ability, especially for protein tyrosine phosphorylation and motility, supporting the fact of the SVA's suppression of sperm sAC activity.

Two possible ways were hypothesized for the SVA's suppression of $[Ca^{2+}]_i$ of mouse sperm. The SVA either blocks calcium influx or enhances intracellular calcium clearance in sperm. The contention that the SVA may alter intracellular calcium clearance is supported by the finding that the SVA decreased $[Ca^{2+}]_i$ in EDTA-containing Ca^{2+}-free culture medium (Fig. 4). In this respect, PMCA might be involved in the SVA's suppression of $[Ca^{2+}]_i$ as evidenced by the following observations: first, the SVA decreased both $[Ca^{2+}]_i$ and pH_i in sperm (Fig. 4); and second, the suppressive effect of the SVA on $[Ca^{2+}]_i$ was gradually reduced by increasing the concentration of CE (a PMCA inhibitor) (Fig. 5B). The inverse correlation between the SVA's effect and the CE concentration

strongly supports the role of PMCA in the SVA's suppression ability of $[Ca^{2+}]_i$.

PMCA plays an important role in the fine control of the cytosolic free Ca^{2+} concentration and intracellular Ca^{2+} homeostasis. This plasma membrane Ca^{2+}-ATPase is concentrated 18- to 25-fold in the caveolae/raft membranes which are abundant in sphingolipids, cholesterol, and gangliosides [Fujimoto, 1993; Schnitzer et al., 1995]. SPM/phospholipids are abundant in lipid rafts and are believed to be regulators of PMCA in caveolae [Pang et al., 2005]. In sperm, PMCA was located at the apical acrosome [Schuh et al., 2004], the principle piece of sperm [Wennemuth et al., 2003; Schuh et al., 2004], and postacrosomal segment [Adeoya-Osiguwa and Fraser, 1996]. However, Wennemuth et al.'s [2003] report showed that PMCA was strictly in the principle piece, and Adeoya-Osiguwa and Fraser's [1996] results showed that PMCA in postacrosomal segment is intracellular. Another Ca^{2+} efflux channel, NCX, is located at the sperm midpiece and flagellum [Vines et al., 2002]. In line with Schuh et al.'s [2004] localization of PMCA and Vines et al.'s [2002] localization of NCX, the SVA binds to the apical acrosome and midpiece of mouse sperm [Huang et al., 1999]. This result strongly supports that the decapacitation effect of the SVA on physiological $[Ca^{2+}]_i$ may occur through regulation of PMCA activity.

Our previous results showed the binding of the SVA with choline-containing phospholipids, for example, PC and SPM [Huang et al., 1999]. Based on tryptophan fluorescence spectral analyses, the K_d of SPM–SVA was determined to be $\sim$3 μM which is much higher than that of PC-SVA (91 μM) [Fig. 6; Huang et al., 1999]. The K_d of SPM–SVA was closer to the IC_{50} (>2 and <20 μM) of the SVA's suppressive effect on BSA- and PAF-induced capacitation signals in mouse sperm [Huang et al., 2005, 2007]. The potential for SPM to modulate sperm capacitation signaling is supported by the recent observation that sphingomyelinase (SPMase) treatment induces capacitation-associated protein tyrosine phosphorylation in boar sperm [Shadan et al., 2004]. In our experiments, the fact that the SVA bound to SPM with a higher affinity than to PC implies the potential targeting of the SVA toward SPM/lipid rafts in plasma membranes to regulate PMCA activity. This hypothesis is strongly supported by a recent finding of Kawano et al. [2008] who demonstrated that ganglioside GM1 in lipid rafts is able to mediate decapacitation effects of seminal plasma protein SVS2 in murine spermatozoa. In addition, proteins in lipid rafts of mouse sperm plasma membranes, such as bovine seminal vesicle secretory protein 7 (caltrin, a calcium transport inhibitor), were identified, and their decapacitation regulation through lowering $[Ca^{2+}]_i$ during ejaculation was demonstrated [Sleight et al., 2005]. These results strongly support the decapacitation regulation of seminal plasma protein in lipid rafts of sperm.

PMCA was also demonstrated to be the major calcium pump ensuring lower $[Ca^{2+}]_i$ in mouse and human sperm [DasGupta et al., 1994; Adeoya-Osiguwa and Fraser, 1996; Wennemuth et al., 2003]. It was linked to sperm motility and fertility [Schuh et al., 2004]. The regulation of sperm PMCA activity by seminal plasma protein is strongly supported by recent reports which demonstrated that bovine sperm PMCA is stimulated by PDC-109 (BSP-A1/A2), a secretory protein of bovine seminal vesicles, to remove Ca^{2+} from sperm and enhance capacitation [Sánchez-Luengo et al., 2004; Triphan et al., 2007]. Similar to the SVA, bovine seminal plasma proteins (PDC-109, BSP-A3, and BSP-30-kDa) share the same characters of being abundantly secreted by seminal vesicles and binding to phospholipids. Different from the SVA in suppression, BSP proteins show activation of sperm capacitation. Upon ejaculation, BSP proteins bind to choline phospholipids of sperm membranes, and stimulate phospholipid and cholesterol efflux from sperm membranes [Thérien et al., 1998]; however, with the similar character of choline phospholipid binding (SPM and PC), the SVA showed a negative effect on GM1 binding dynamics in BSA-treated sperm (Fig. 7), suggesting that the SVA did not affect the cholesterol distribution of sperm membranes. The distinct mechanisms between the SVA and BSP still are not well understood.

The SVA shows suppression of BSA-/CD- and PAF-induced mouse sperm capacitation. BSA and CD are thought to remove the membranous cholesterol which induces the capacitation process in mouse sperm [Go and Wolf, 1985; Visconti et al., 1995a, 1999]. In contrast, PAF is known to induce mouse sperm capacitation by disturbing the membrane or interacting with the PAF receptor [Roudebush et al., 2005; Huang et al., 2007]. Thus, a unique mechanism of the SVA must exist which differs from cholesterol removal for its suppressive ability. This concept was also supported by the fact that the SVA did not alter the lipid raft distribution pattern under capacitation conditions (Fig. 7). In our study, interactions between the SVA and SPM on plasma membranes may affect the PMCA activity in lipid rafts which resulted in enhanced Ca^{2+} efflux and a decrease in $[Ca^{2+}]_i$. Since calcium is known to be a key regulator mediating capacitation-related signals, a decrease in $[Ca^{2+}]_i$ in sperm would suppress the activation of sAC, PKA, and calcium dependent protein kinases, leading to the suppression of protein tyrosine phosphorylation and capacitation. This suppressive effect of the SVA on capacitation may enable sperm to avoid unfruitful capacitation until conditions are appropriate (in the uterus or oviduct where the egg resides and the concentration of the SVA is low).

ACKNOWLEDGMENTS

We thank Professor Jung S. Huang at San Louis University for the technique supporting and encouragements.

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