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Suppression Effect of Seminal Vesicle Autoantigen on Platelet-Activating Factor-Induced Mouse Sperm Capacitation

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Abstract Mammalian sperm gain the ability to fertilize an egg successfully by the capacitation process. An unregulated capacitation process causes sperm to undergo a spontaneous acrosome reaction (AR) and resulting in loss of their fertilization activity. Thus, functional sperm activation is tightly regulated by a capacitation and suppression (decapacitation) mechanism. Factors, such as platelet-activating factor (PAF) present in both sperm and the female genital tract, are able to stimulate sperm capacitation. Seminal plasma is thought to have the ability to suppress sperm capacitation; however, the regulatory mechanisms of seminal plasma protein on sperm capacitation are not well understood. Recently, we demonstrated that seminal vesicle autoantigen (SVA), a major seminal vesicle secretory protein, is able to suppress mouse sperm capacitation. To further study the suppression spectra of SVA on sperm capacitation, we investigated the effect of SVA on PAF-induced mouse sperm capacitation-related signals. Here, we demonstrate that SVA decreases the $[Ca^{2+}]_i$ to suppress the PAF's effects on $[Ca^{2+}]_i$, the cAMP level, protein tyrosine phosphorylation, and capacitation. The inhibition of PAF-induced protein tyrosine phosphorylation and capacitation by SVA can be reversed by cAMP agonists. Characterization of the interactions of SVA with PAF by TLC overlay and tryptophan fluorescence spectrum analyses indicates that SVA is capable of binding PAF with an apparent dissociation constant $K_d > 50$ μM. Together with these results, we demonstrate that SVA deceases $[Ca^{2+}]_i$ and cross-talks with PAF-induced intracellular signals to regulate mouse sperm capacitation. J. Cell. Biochem. 100: 941–951, 2007.

Key words: capacitation; seminal plasma protein; sperm

Mammalian sperm produced in the testis are first immobile and acquire the ability to swim forward during their transport into the epididymis. Following ejaculation, sperm become motile, yet lack fertilizing competence. Sperm gain fertilization capacity after residence in the female genital tract for a finite period of time. The physiological changes of sperm during this period are collectively called "capacitation." Capacitation is a complex process first described and defined independently by Chang [1951, 1955] and Austin [1951, 1952]. It occurs in the oviduct or uterus, depending on the species [Yanagimachi, 1994]. The capacitation process involves alteration of the sperm plasma membrane, elevation of the intracellular calcium concentration ($[Ca^{2+}]_i$) and the cAMP level, an increase of intracellular pH (pH_i), and stimulation of protein tyrosine phosphorylation [Yanagimachi, 1994; Visconti and Kopf, 1998]. It leads to energy consumption, hypermotility, and eventually to the acrosome reaction (AR) of sperm [Yanagimachi, 1994].

Several factors play important roles in sperm capacitation and fertilization. These include serum albumin (which is abundant in the female

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reproductive tract) and PAF (platelet-activating factor, 1-*O*-alkyl-2-*O*-acetyl-sn-glycero-3-phosphoryl-choline, which is present in both sperm and the female reproductive tract). Unlike serum albumin which is believed to remove cholesterol from sperm membranes to induce capacitation signals [Go and Wolf, 1985; Visconti and Kopf, 1998], PAF is thought to promote sperm capacitation via disturbing the sperm plasma membrane [Huo and Yang, 2000] or PAF-PAF receptor-mediated signaling [Roudebush, 2001; Roudebush et al., 2005]. The presence of PAF in sperm has been reported in many mammalian species [Roudebush, 2001; Roudebush and Diehl, 2001; Wu et al., 2001; Roudebush et al., 2005]. The level of endogenous PAF is positively correlated with the fertilization potential [Roudebush, 2001; Toledo et al., 2003; Roudebush et al., 2005]. It has been reported that PAF is secreted from sperm to interact with membrane phospholipid components [Huo and Yang, 2000] and induce sperm capacitation in an autocrine manner [Wu et al., 2001]. Exogenous PAF has been shown to induce sperm capacitation and the AR in many species [Fukuda et al., 1994; Krausz et al., 1994; Huo and Yang, 2000; Odeh et al., 2003; Kumar and Sharma, 2005]. In mammalian sperm, PAF was hypothesized to regulate cellular functions by affecting $[Ca^{2+}]_i$ and inositol triphosphate (IP_3) levels [Roudebush, 2001; Roudebush et al., 2005]. In addition, PAF was independently demonstrated to stimulate protein kinase [Sengoku et al., 1996], enhance sperm motility (straight-line velocity, VSL) [Wang et al., 1994], and protein tyrosine phosphorylation [Luconi et al., 1995] in human sperm.

Successful fertilization is tightly regulated by capacitation and suppression factors. The inhibition of capacitation by suppression factors is referred to as "decapacitation" [Chang, 1957]. Several decapacitation factors have been detected in the epididymis and seminal plasma [Parks and Hough, 1993; Fraser, 1998; Lopes et al., 1998; Mortimer et al., 1998; Villemure et al., 2003]. However, the regulatory mechanisms of these decapacitation factors have not been well defined. Recently, we have demonstrated that a seminal plasma protein, seminal vesicle autoantigen (SVA), is able to bind choline-containing phospholipids, such as sphingomyelin (SPM) and phosphatidylcholine (PC), to suppress bovine serum albumin (BSA)-induced capacitation signals [Huang et al., 1995, 1999, 2000, 2005]. In an effort to better understand SVA-mediated suppression on sperm capacitation, we demonstrate in this communication that SVA cross-talks with PAF-stimulated intracellular signals to regulate mouse sperm capacitation. The molecular events associated with SVA-mediated suppression of PAF activities include lowering $[Ca^{2+}]_i$ and the cAMP level, suppressing protein tyrosine phosphorylation and capacitation. The effects of SVA on PAF-induced protein tyrosine phosphorylation and capacitation can be reversed by cAMP agonists.

MATERIALS AND METHODS

Materials

BSA free from fatty acid, chlortetracycline (CTC), 3-isobutyl-1-methylxanthine (IBMX), PC, SPM, Lyso-phosphatidylcholine (Lyso-PC), platelet-activating factor (PAF, 1-*O*-alkyl-2-*O*-acetyl-sn-glycero-3-phosphoryl-choline), phosphatidic acid (PA), Lyso-platelet-activating factor (Lyso-PAF), polyvinylalcohol, phosphomolybdic acid spray, and Dragendorff reagent were from Sigma (St. Louis, MO). Aluminum-backed silica gel thin layer chromatogram plates (TLC) were from Whatman (Maidstone, UK). Anti-phosphotyrosine monoclonal antibody (clone 4G10) was from UBI (Lake Placid, NJ), and horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody was from Jackson ImmunoResearch Lab (West Grove, PA). Percoll, chemiluminescence detection ECL plus, PD-10 columns, and cAMP assay kits (RPN 225) were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Poly (isobutyl methacrylate) was from Aldrich (Milwaukee, WI), ditutylryl cAMP (dbcAMP) and Rp-cAMPS were from Research Biochemicals International (Natick, MA), H-89 (N-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide) was from LC Laboratories (Woburn, MA), fluo-3 AM was from Molecular Probes (Eugene, OR), and propidium iodide (PI) was from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Sperm Preparation and CTC Assay

Outbred CD-1 mice purchased from Charles River Laboratories (Wilmington, MA) were bred in the Animal Center at Taipei Medical University College of Medicine. 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., 2005]. Polyvinylalcohol (1 mg/ml) was added to the medium to serve as a sperm protectant. Mature mouse sperm were harvested by a swim-up procedure from the caudal epididymis and isolated with a 20–80% Percoll gradient by centrifugation at 275*g* at room temperature for 30 min. Sperm viability was determined by counting damaged spermatozoa stained by propidium iodide (PI). The viability and progressive motility of the sperm fraction used in the present study were more than 90%. The population of the capacitated stage in mouse sperm was analyzed by the CTC staining method as described previously [Ward and Storey, 1984].

Preparation of Biotinylated SVA and PAF

SVA was purified and biotinylated SVA was prepared as described previously [Huang et al., 1999]. PAF was dissolved in chloroform/methanol (1:1, v/v) as a 10 mM stock solution and stored at −20°C. For sperm culture, a 20-fold PAF solution (200 μM) was prepared by evaporating the organic solvent of the stock solution under nitrogen and then the residues were dissolved in distilled water in a siliconized eppendorf tube. The 200 μM PAF solution was sonicated and diluted with HM to the final working solution (10 μM) before use. Siliconized eppendorf tubes were used throughout the experiments to prevent adherence of PAF to the eppendorf tubes which would affect the final concentration.

Detection of Protein Tyrosine Phosphorylation

Sperm (5×10^6 cells/ml) were incubated at 37°C for 90 min under different experimental conditions, with PAF (0–10 μM) in the presence SVA (0–200 μM). In some experiments dbcAMP (a cAMP agonist, 1 mM) plus IBMX (a phosphodiesterase inhibitor, 100 μM), H-89 (a PKA inhibitor, 0–30 μM), or Rp-cAMP (a cAMP antagonist, 1 mM) were added. Cell lysates were prepared, and subjected into a 10% SDS–PAGE gel, and then transferred to a PVDF membrane for Western blot analysis as described previously [Huang et al., 2005]. 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.

cAMP Assay

Sperm cells were incubated in IBMX (100 μM)-containing HM medium supplemented with or without PAF (10 μM) and SVA (0–66 μM) at 37°C for 30 min. After incubation, sperm cells were harvested and the intracellular cAMP levels in living sperm were detected using a non-radioactive enzyme immunoassay kit (RPN 225, Amersham Pharmacia) according to the manufacturer's instructions.

Flow Cytometry

Sperm $[Ca^{2+}]_i$ was determined using fluo-3 AM as described previously [Huang et al., 2005]. The fluo-3 AM-loaded sperm (10^6 cells/ml) were treated with SVA (0–66 μM) in the presence or absence of PAF (10 μM) and analyzed by flow cytometry (FACScan, BD). The fluorescence intensity of sperm was quantified for 10,000 individual sperm cells.

Fluorescence Spectra

The fluorescence intensity of SVA in tris-buffered saline (TBS) at pH 7.4 was measured and expressed in arbitrary units using a Hitachi F-4500 fluorescence spectrophotometer according to our previous report [Huang et al., 1999]. The fluorescence intensity at wavelength λ_2 (nm) while the fluorophore was excited at wavelength λ_1 (nm) is denoted by $F^{\lambda_2}_{\lambda_1}$. A modified Scatchard plot [Epstein et al., 1974] was constructed to analyze the fluorescence data of a complex formed by SVA and PAF:

$$|\Delta F|/[L]_{free} = \Delta F_\infty/K_d - |\Delta F|/K_d$$

where ΔF is the change in 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 $[L]_{total}$.

TLC-Overlay Technique

Binding of biotinylated SVA to phospholipids on TLC plates was performed as described previously [Huang et al., 1999]. In brief,

purified lipids were chromatographed on the TLC plates in chloroform/methanol/H_2O (65:25:4, by vol.). The plates were air-dried and immersed in 0.1% poly (isobutyl methacrylate) in hexane for 1 min. The chromatograms were dried and blocked in 5% (w/v) non-fat skim milk/PBS, and were overlaid with biotinylated SVA (2 μg/ml) in blocking buffer (100 μl/cm^2) at 25°C for 90 min. After washing processes, the HRP-conjugated streptavidin (1:1,000 dilution) and ECL system were used to monitor the binding of biotinylated SVA on lipids. The lipids on triplicate plates were detected by spraying with a phosphomolybdic acid solution or Dragendorff reagent [Huang et al., 1999].

Statistical Analysis

All experiments were repeated at least three times with three different pools of sperm samples from four or five male mice. The data were expressed as the mean ± SD. Difference in means was assessed by one-way analysis of variance (ANOVA), followed by the Tukey–Kramer multiple comparisons test (GraphPad Instat3 program).

RESULTS

SVA Suppresses PAF-Induced Mouse Sperm Capacitation

When mature sperm are ejaculated from the caudal epididymis and move to the female reproductive tract, they interact with suppression factors (in the seminal plasma) and capacitation factors (in the epididymis, for example, PAF, and in the female reproductive tract, for example, PAF and serum albumin). SVA is a major secretory protein in semen with a concentration of ~300 μM. It suppresses BSA-induced capacitation signaling in mouse sperm [Huang et al., 2000, 2005]. Since PAF is synthesized and secreted by sperm and promotes fertilization in several species [Kumar et al., 1988; Huo and Yang, 2000; Roudebush and Diehl, 2001], we hypothesized that SVA may play a role in regulating of PAF-induced capacitation in mouse sperm. To test this hypothesis, we firstly examined the capacitation status of caudal epididymis sperm treated with increasing concentrations of PAF. As shown in Figure 1A, PAF induced mouse sperm capacitation in a dose-dependent manner. At 10 μM, PAF induced capacitation in 45 ± 8% of sperm. BSA (0.3%) was used to serve as a positive control; it induced capacitation in around 55% of sperm (data not shown). Fewer than 15% of sperm underwent capacitation in the absence of PAF with or without SVA. Lyso-PAF (up to 10 μM) failed to induce capacitation (data not shown), suggesting the specific activity of PAF on mouse sperm. SVA suppressed PAF-induced capacitation in mouse sperm (Fig. 1B). In our results, SVA significantly suppresses the PAF-induced sperm capacitation at

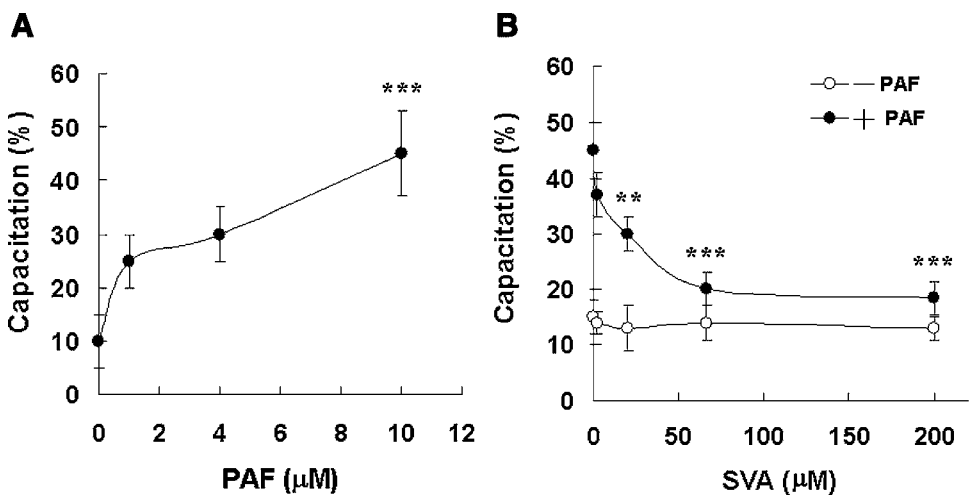

Fig. 1. SVA-mediated suppression of PAF-induced sperm capacitation. **A**: Mouse sperm were treated with increasing concentrations of PAF (1–10 μM). **B**: Mouse sperm were treated with increasing concentrations of SVA (0–200 μM) ± 10 μM PAF. After 90 min, the percentage of sperm undergoing capacitation was determined using the CTC fluorescence method. The mean ± SD of five independent determinations for each condition is shown. ***denotes that the difference from samples without PAF exposure (0 μM) is significant with $P < 0.001$ (A); **denotes that the difference from PAF treatment in the absence of SVA (0 μM) is significant with $P < 0.01$; and ***with $P < 0.001$ (B).

20–66 μM, whose concentration is well below its physiological concentration in semen.

SVA Suppresses the PAF's Effects on $[Ca^{2+}]_i$ and the cAMP Level in Mouse Sperm

We have previously demonstrated that SVA is able to suppress the BSA-induced $[Ca^{2+}]_i$ and capacitation in mouse sperm [Huang et al., 2005]. Given that SVA is capable of suppressing PAF-induced capacitation, we determined the effect of SVA on $[Ca^{2+}]_i$ in mouse sperm stimulated by PAF. As shown in Figure 2A, 10 μM PAF elevated $[Ca^{2+}]_i$ to 118 ± 5% relative to the control group ($P < 0.001$). SVA suppressed $[Ca^{2+}]_i$ with or without PAF in a dose-dependent manner (Fig. 2B). The half-maximum concentration (IC_{50}) of SVA to suppress PAF-induced elevation of $[Ca^{2+}]_i$ was estimated to be less than 10 μM. The effect of BSA (0.3%) on $[Ca^{2+}]_i$ was used as a positive control. It increased $[Ca^{2+}]_i$ to 153 ± 6% (data not shown).

Since Ca^{2+} is one of the activators to stimulate adenylate cyclase [Visconti and Kopf, 1998], the effect of SVA on PAF-induced elevation of the cAMP level was determined. As shown in Figure 3A, 10 μM PAF increased the cAMP level from 2.5 to 3.0 pmole/10^6 cells ($P < 0.01$). SVA suppressed both the PAF-induced elevation as well as the basal level of cAMP in a dose-dependent manner (Fig. 3B). The effect of BSA on the cAMP level was used as a positive control. BSA (0.3%) increased the cAMP level to 3.9 pmole/10^6 cells ($P < 0.001$, data not shown).

A cAMP Agonist Reverses SVA's Suppression of PAF-Induced Protein Tyrosine Phosphorylation and Capacitation in Mouse Sperm

Protein tyrosine phosphorylation positively correlates with changes in BSA-induced mouse sperm capacitation [Visconti et al., 1995a,b]. Given that PAF is able to enhance capacitation of mouse sperm (Fig. 1), we then determined the effect of PAF on protein tyrosine phosphorylation. As shown in Figure 4A, two proteins of M.W. ~120 and ~60 kDa were found to be tyrosine-phosphorylated in control sperm with normal motility (lane 1). PAF (10 μM) effectively stimulated tyrosine phosphorylation of several proteins of M.W. ~50–100 kDa and a protein of M.W. <50 kDa (lane 4). The tyrosine phosphorylation of a 120 kDa protein (p95/106 hexokinase) did not respond to PAF or BSA stimulation as reported previously [Kalab et al., 1994]. The pattern of tyrosine-phosphorylated proteins in sperm treated with PAF was virtually similar to that treated with BSA except with weaker intensity (lane 4 vs. 5)

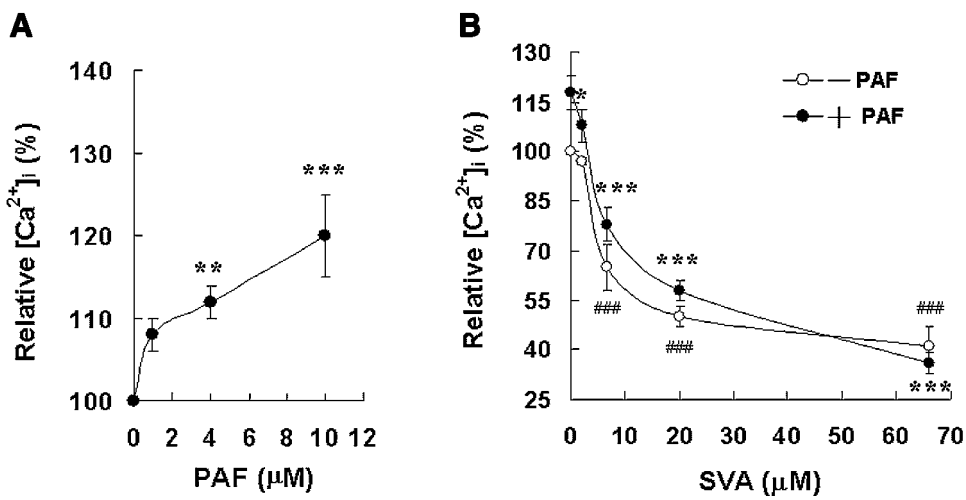

Fig. 2. SVA-mediated suppression of PAF-induced elevation of $[Ca^{2+}]_i$ in mouse sperm. **A**: Fluo-3 AM-loaded sperm were treated with increasing concentrations of PAF (1–10 μM). **B**: Fluo-3 AM-loaded sperm were treated with increasing concentrations of SVA (0–66 μM) ± 10 μM PAF. After 90 min, sperm were collected, and the fluorescence intensity was analyzed by flow cytometry. Relative $[Ca^{2+}]_i$ is shown with $[Ca^{2+}]_i$ in sperm samples without PAF or SVA treatment designated as 100%. The mean ± SD of five independent determinations for each condition is shown. ** denotes that the difference from samples without PAF exposure (0 μM) is significant with $P < 0.01$; and ***with $P < 0.001$ (A); *denotes that the difference from PAF treatment in the absence of SVA (0 μM) is significant with $P < 0.05$, and *** with $P < 0.001$; and ### denotes that the difference from samples without PAF treatment and in the absence of SVA is significant with $P < 0.001$ (B).

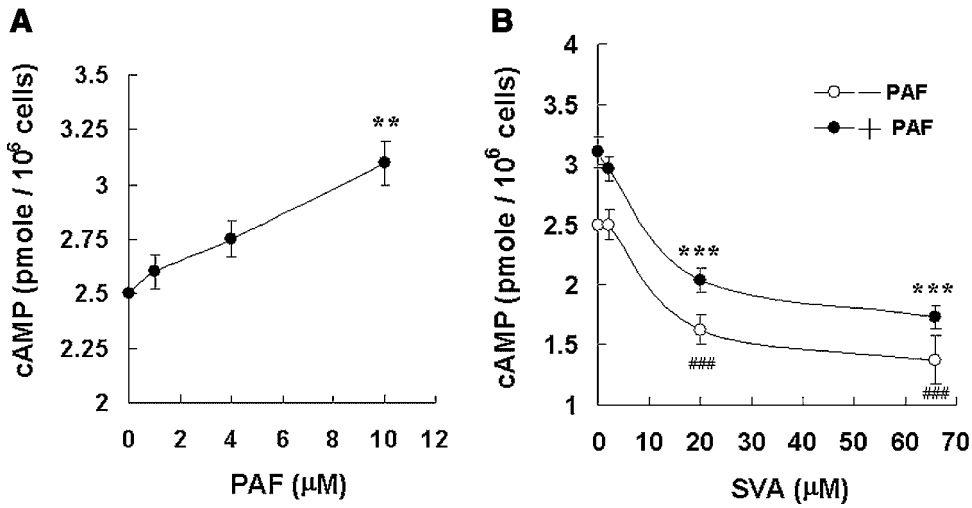

Fig. 3. SVA-mediated suppression of PAF-induced elevation of cAMP level in mouse sperm. Mouse sperm were treated with increasing concentrations of PAF (1–10 μM) (**A**) or with SVA (0–66 μM) ± 10 μM PAF (**B**). After 90 min incubation, sperm were collected and the total cell lysate was extracted to detect the whole amount of intracellular cAMP. The mean ± SD of five independent determinations for each condition is shown. **denotes that the difference from samples without PAF exposure (0 μM) is significant with $P < 0.01$ (A); ***denotes that the difference from PAF treatment in the absence of SVA is significant with $P < 0.001$; and ###denotes that the difference from samples without PAF treatment and in the absence of SVA is significant with $P < 0.001$ (B).

[Visconti et al., 1995a; Huang et al., 2005]. As a negative control, Lyso-PAF (10 μM) did not stimulate tyrosine phosphorylation of these proteins (lane 6).

PKA is involved in BSA-induced protein tyrosine phosphorylation in mouse sperm [Visconti et al., 1995b; Huang et al., 2005]. To validate whether PKA acts upstream of PAF-stimulated protein tyrosine phosphorylation, we determined the effects of H-89, a PKA inhibitor [Chijiwa et al., 1990], and Rp-cAMP, a cAMP antagonist [Botelho et al., 1988], on PAF-induced protein tyrosine phosphorylation. As shown in Figure 4B, H-89 inhibited the PAF-induced tyrosine phosphorylation of proteins of M.W. ~50–100 kDa in a dose-dependent manner with an IC_{50} of ~1 μM. Rp-cAMP also blocked protein tyrosine phosphorylation induced by PAF (Fig. 4C). These results suggest that the PKA-mediated signals may be involved in PAF-induced protein tyrosine phosphorylation in mouse sperm.

Both $[Ca^{2+}]_i$ and cAMP are involved in capacitation-associated protein tyrosine phosphorylation [Visconti et al., 1995a,b; Visconti and Kopf, 1998]. Given that SVA significantly decreased both $[Ca^{2+}]_i$ and the cAMP level, we determined the effect of SVA on PAF-induced sperm protein tyrosine phosphorylation. As shown in Figure 4D, SVA suppressed PAF-induced protein tyrosine phosphorylation in a dose-dependent manner with an IC_{50} of >2 μM and <20 μM. SVA almost completely suppressed protein tyrosine phosphorylation induced by PAF at 66 μM (lane 5), a concentration which is well below the physiological concentration of SVA in semen.

A cAMP analog has been reported to induce protein tyrosine phosphorylation and capacitation in mouse sperm [Visconti et al., 1995b]. In our experiment, we determined the effect of dbcAMP (a cAMP agonist) plus IBMX (inhibitor of cyclic nucleotide phosphodiesterase) on SVA-mediated suppression of PAF activity. As shown in Figure 4D, the suppressive effect of SVA on PAF-induced protein tyrosine phosphorylation was prevented by dbcAMP plus IBMX (lane 7). Furthermore, the cAMP agonist also reversed SVA-mediated suppression of PAF-induced capacitation in mouse sperm (Fig. 4E). These results suggest that SVA acts on the upstream of cAMP-regulated signaling to mediate the suppression of PAF action.

SVA Interacts With PAF

In previous reports, we demonstrated that SVA binds choline-containing sperm phospholipids and suppresses mouse sperm motility and capacitation stimulated by BSA [Huang et al., 1999, 2000, 2005]. Since PAF is a choline-containing phospholipid, we determined the interaction of SVA with PAF by TLC overlay

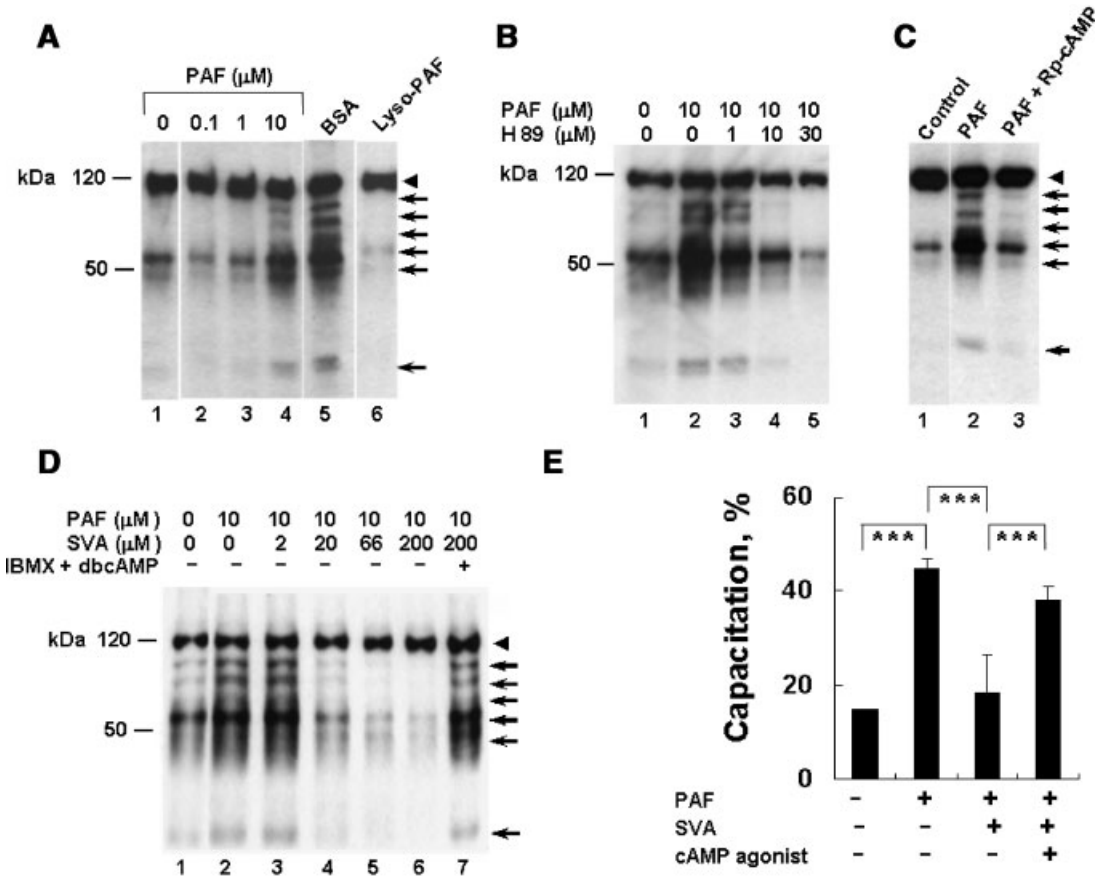

Fig. 4. cAMP agonist reverses the SVA-mediated suppression of PAF-induced protein tyrosine phosphorylation and capacitation in mouse sperm. **A–D**: Detection of protein tyrosine phosphorylation in mouse sperm by Western blot analysis. A: Mouse sperm were incubated under different experimental conditions to induce protein tyrosine phosphorylation: PAF (**lanes 1–4**); BSA (0.3%) (**lane 5**); Lyso-PAF (**lane 6**). B and C: Effects of increasing concentrations of H-89 (0–30 μM) and Rp-cAMP (1 mM) on PAF (10 μM)-induced protein tyrosine phosphorylation in mouse sperm. The effect of BSA (0.3%) is also shown. D: SVA-mediated suppression of PAF-induced protein tyrosine phosphorylation in mouse sperm. Note the reversal of SVA-mediated suppression by dbcAMP plus IBMX (**lane 7**). The arrows indicate the location of capacitation-related tyrosine-phosphorylated proteins, and the arrowhead denotes the location of a 120 kDa tyrosine-phosphorylated protein unrelated to capacitation. **E**: Percentage capacitation of mouse sperm as determined by the CTC fluorescence method. The mean ± SD of three independent determinations for each condition is shown. ***denotes that the difference from samples under a different experimental condition (as indicated) is significant with $P < 0.001$.

binding analysis. As shown in Figure 5A, PA and phosphocholine-containing lipids, including PC, SPM, Lyso-PC, PAF, and Lyso-PAF were chromatographed on a TLC plate and detected with phosphomolybdic acid spray to identify the phospholipid characters (Fig. 5A, Panel a). Choline-containing phospholipids were specifically stained with the Dragendorff reagent (Panel b). The TLC-overlay binding analysis revealed that biotinylated SVA bound PC, Lyso-PC, SPM, PAF, and Lyso-PAF, but did not interact with PA (Panel c). These results confirm our previous report that SVA is capable of binding choline-containing phospholipids [Huang et al., 1999], including PAF.

To further characterize the SVA interaction with PAF, we determined the binding affinity of SVA for PAF by analyzing changes of the SVA tryptophan fluorescence spectrum when SVA was titrated with PAF. SVA contains two tryptophan residues per molecule: one is located on the protein surface and the other is embedded in the folding structure. The tryptophan residue on the protein surface is restricted to a configuration which differs from that of free tryptophan according to their fluorescence characterization [Huang et al., 1995]. Figure 5B displays the emission spectra of SVA in the presence and absence of PAF. The presence of 5.0% (v/v) ethanol in the protein solution did not

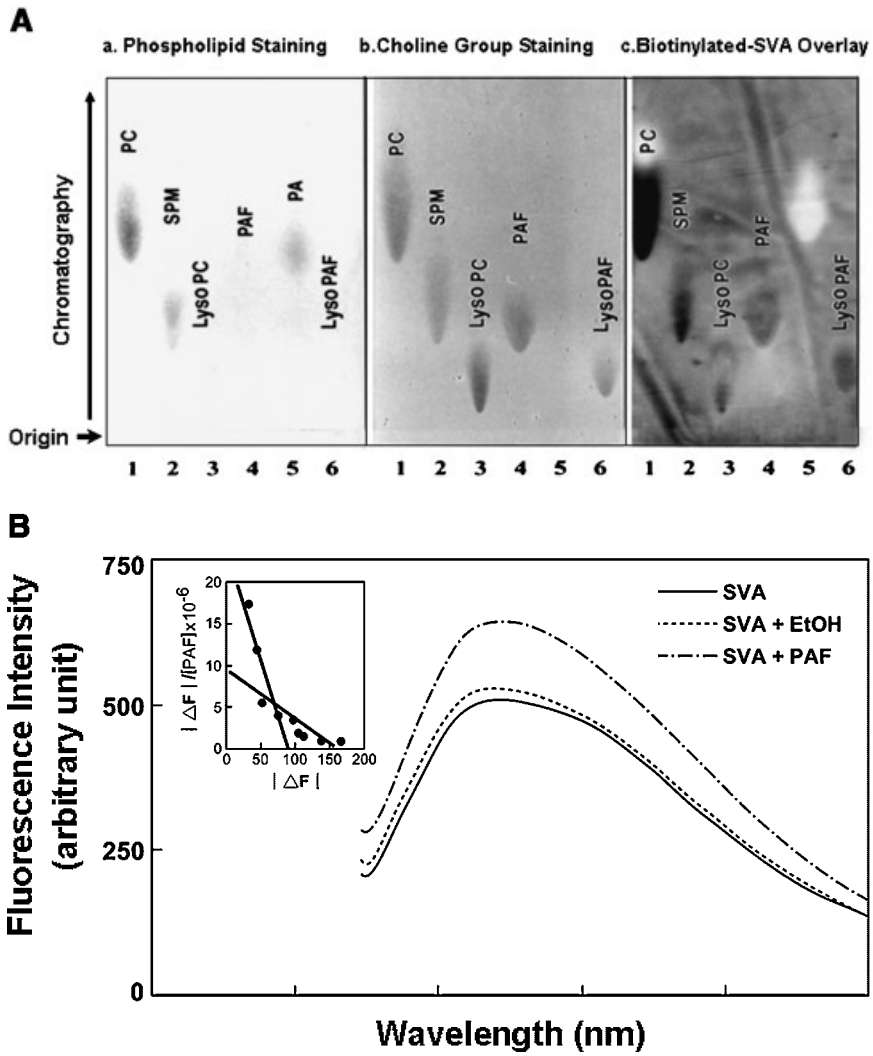

Fig. 5. Analysis of the interaction of SVA with PAF using TLC and tryptophan fluorescence spectrometry. **A**: Purified phospholipids (30 μg) were spotted and chromatographed on TLC plates in chloroform/methanol/water (65:25:4, by vol.). Phospholipids were detected with phosphomolybdic acid spray (**a**), and choline-containing phospholipids were detected with Dragendorff reagent spray (**b**). Binding of biotinylated SVA for a separated sample of phospholipids using a TLC-overlay binding technique is also shown (**c**). Abbreviations: PC, phosphatidylcholine; SPM, sphingomyelin; Lyso-PC, Lyso-phosphatidylcholine; PAF, platelet-activating factor; PA, phosphatidic acid; Lyso-PAF, Lyso-platelet-activating factor. **B**: Emission spectra of SVA (6.5 μM) ± PAF (40 μM) was scanned with an excitation wavelength of 295 nm. The emission spectra of SVA protein in TBS is expressed as the solid line; 5% (v/v) ethanol in the SVA protein solution is shown as the dotted line, and 40 μM PAF in 1% (v/v) ethanol plus SVA protein is shown as the dot-dash line. The modified Scatchard plot for the binding of PAF to SVA is given in the inset. The correlation coefficient was calculated to be greater than 0.98.

alter the protein fluorescence in either the spectral profile or its emission intensity (dotted line vs. solid line). PAF (40 μM) greatly enhanced the protein fluorescence intensity without shifting the emission peak (dot-dash line vs. solid line). Apparently, interaction of SVA with PAF resulted in an enhancement in SVA tryptophan fluorescence. The SVA–PAF interaction was probed by analyzing the ability of PAF to perturb SVA protein fluorescence. We obtained $F_{\lambda_1}^{\lambda_2}$ values by titrating the SVA protein solution (6.5 μM) with PAF and calculated the dissociation constant as described previously [Epstein et al., 1974]. As shown in Figure 5B (inset), the modified Scatchard plot curve indicated that there were two types of PAF-binding sites on the SVA protein molecule. The apparent K_ds of the high-affinity and low-affinity binding sites were estimated to be 52.5 μM and 357 μM, respectively. The apparent K_ds of SVA binding to PAF were well above the effective concentrations required for SVA to

suppress the PAF's effects on $[Ca^{2+}]_i$, the cAMP level, protein tyrosine phosphorylation, and capacitation in mouse sperm. This result suggests that SVA-mediated suppression of PAF activity may be more than a scavenging effect. This contention is supported by the observation that a 1,000-fold excess of PAF failed to completely block SVA binding to mouse sperm (data not shown).

DISCUSSION

PAF is a putative capacitation factor to stimulate sperm capacitation and fertilization. Exogenous PAF is thought to affect sperm motility and fertilization with unclear mechanism. In our study, with recent advanced assay systems, we demonstrate that PAF affects $[Ca^{2+}]_i$, the cAMP level, protein tyrosine phosphorylation, and capacitation in mouse sperm. The PAF-induced elevation of $[Ca^{2+}]_i$ and the cAMP level is low but significant (Figs. 2 and 3). The small increase of the cAMP level induced by PAF is somewhat similar to that of the previous report [Wang et al., 1994]. PAF-induced cAMP is lower than the BSA's action and this is in coincident with the weaker protein tyrosine phosphorylation induced by PAF than BSA (Fig. 4A, lane 4 vs. 5). Besides, the PAF-induced protein tyrosine phosphorylation was suppressed both by Rp-cAMP and H-89 (Figs. 4B,C), suggesting that PKA may involve in PAF actions. In this study, PAF stimulates protein tyrosine phosphorylation and capacitation in mouse sperm at 10 μM concentration with which sperm maintain their viability and motility. We rule out the non-specific effects of PAF on mouse sperm capacitation because Lyso-PAF, which was at the same concentration as PAF, did not stimulate capacitation and capacitation-associated protein tyrosine phosphorylation (Fig. 4A, lane 6). Moreover, the PAF concentration (10 μM) used in these experiments is close to that of the previous report which demonstrated PAF (18 μM) significantly stimulates mouse sperm motility in vitro [Wu et al., 2001]. We did not detect significant protein tyrosine phosphorylation or capacitation in mouse sperm treated with 1 μM PAF as reported previously [Huo and Yang, 2000] (Fig. 4A, lane 3). The divergent results may be due to use of different strains of mice and/or different culture conditions used for the capacitation assay.

Unregulated capacitation processes direct sperm to undergo unfruitful capacitation and a spontaneous AR. Seminal plasma has been reported to contain a high concentration of suppression factors (decapacitation factors) to prevent pre-hyperactivation of sperm. However, the active components and regulatory mechanisms of the seminal plasma proteins still remain unclear. In the present study, SVA, as a component of seminal plasma, significantly decreases both the PAF-induced as well as the basal $[Ca^{2+}]_i$ and the cAMP level, and suppresses the PAF-induced protein tyrosine phosphorylation and capacitation. A cAMP agonist (dbcAMP) plus IBMX effectively overcome the SVA's suppression of protein tyrosine phosphorylation and capacitation, suggesting that SVA acts upstream of cAMP-mediated signaling. Besides, SVA significantly suppressed $[Ca^{2+}]_i$. Given that Ca^{2+} is thought to be one of the upstream regulators of capacitation signaling, the decrease of $[Ca^{2+}]_i$ seems to play a role in the SVA suppression effect. In sperm, $[Ca^{2+}]_i$ is regulated either by the extracellular entry through calcium channels or the calcium release from a major intracellular store, the acrosome. The acrosomal vesicle is a calcium store that is sensitive to the IP_3 second messenger pathway in mouse sperm [Herrick et al., 2005]. The IP_3 receptors have been localized to the acrosomes [Walensky and Snyder, 1995], or acrosomes and the neck region [Ho and Suarez, 2001; Levine et al., 2002], while the PAF receptors were expressed at the midpiece and proximal acrosome of mouse sperm [Wu et al., 2001]. The close location of IP_3- and PAF-receptors hints that PAF affect $[Ca^{2+}]_i$. Interestingly, the binding pattern of SVA on mouse sperm [Huang et al., 1999] is somewhat similar to the membrane location pattern of PAF receptors. These observations imply the cross-talk of SVA with PAF-induced sperm capacitation signals, and this thought is emphasized by the fact that SVA suppresses the PAF-induced capacitation (Figs. 1–4). In support of the role of SVA's actions on $[Ca^{2+}]_i$ to suppress the capacitation process, a previous report has been demonstrated that the accessory sexual organ, such as a seminal vesicle, plays a role in maintaining an optimal calcium environment for sperm function [Hong et al., 1984]. This hypothesis is further supported by a seminal vesicle secretory protein, caltrin, is able to interact with sperm to lower $[Ca^{2+}]_i$ during ejaculation [Coronel et al., 1993].

SVA bound choline-containing phospholipids selectively. Phosphocholine-containing lipids make up more than 70% of the total lipid in the plasma membrane of mouse spermatozoa. The phospholipid-binding proteins have been demonstrated to play roles in reproduction. Studies by Manjunath and Therien [2002] suggest that a group of bovine seminal vesicle secretion proteins (bovine seminal plasma, BSP) bind some choline-containing phospholipids and promote efflux of cholesterol and phospholipids to stimulate sperm capacitation. In our results, SVA binds choline-containing phospholipids, such as PC, SPM, PS, PAF, Lyso-PC, and Lyso-PAF. The binding pattern of SVA on choline-containing phospholipids is somewhat similar to that of BSP proteins; however, the biological functions of these two proteins are quite different. SVA suppresses, while BSP stimulates the sperm capacitation process. Comparison of the primary structure of SVA with BSP indicates that these two proteins show no significant similarity to each other [Huang et al., 1999]. Recent studies suggested lipid rafts, which are highly enriched in cholesterol, gangliosides, and sphingolipids, are involved in regulation of sperm capacitation and decapacitation signaling [Sleight et al., 2005]. Given that both SVA and BSP are phospholipid-binding proteins, the different activity of SVA and BSP on sperm capacitation may due to the different action mechanisms in lipid raft of mouse sperm. The mechanism of SVA binding to choline-containing phospholipids to suppress $[Ca^{2+}]_i$ of mouse sperm requires further study. The understanding of mechanisms of capacitation and decapacitation processes in sperm will not only allow effective control of fertilization, but will also aid in functionally resolving causes of infertility.

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