Seminal vesicle autoantigen, a novel phospholipid-binding protein secreted from luminal epithelium of mouse seminal vesicle, exhibits the ability to suppress mouse sperm motility

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Seminal vesicle autoantigen (SVA) is a 19 kDa glycoprotein purified from mouse seminal vesicle secretion. It was quantified to be 0.9 % (w/v) in the seminal vesicle fluid. We examined its distribution in the accessory sexual gland, characterized its binding sites on the sperm surface and assessed its effect on sperm motility. It was immunolocalized on the epithelium of the primary and secondary folds in the tissue. Mouse spermatozoa collected from caudal epididymis were devoid of SVA. A cytochemical study illustrated the presence of SVA-binding region on the entire cells. The cytochemical staining intensity for the binding of SVA to spermatozoa remained even when the cells were pretreated with protease digestion, acid or heat at 100 °C for 10 min. Moreover, the SVA–sperm binding could be inhibited by the dispersed sperm lipid. The specificity of interaction between ^{125}I-SVA and phospholipids was studied by TLC overlay techniques. The radiolabelled protein showed strong binding to purified phosphatidylcholine and phosphatidylserine and weak binding to purified sphingomyelin, lysophosphatidylcholine and phosphatidylethanolamine, but did not interact with phosphatidic acid, lysophosphatidic acid or phosphatidylinositol. Among the lipids extracted from spermatozoa, SVA showed strong binding to phosphatidylcholine and weak binding to sphingomyelin and neutral lipids. The assay for SVA–sperm binding with ^{125}I-SVA determined the IC_{50} as being $(3.89 \pm 0.65) \times 10^{-5}$ M^{-1}, which is compatible with an apparent dissociation constant of $(9.10 \pm 0.02) \times 10^{-5}$ M^{-1} estimated by fitting the data of phosphatidylcholine-perturbed SVA fluorescence to a modified Scatchard plot. SVA showed an ability to suppress sperm motility. The average path velocity, straight-line velocity and curvilinear velocity of sperm were not detectable by computer-assisted sperm assay after incubation of the cells in the presence of 0.3 % SVA at 37 °C for more than 40 min.

Key words: fluorescence, glycoprotein, immunolocalization, reproduction.

INTRODUCTION

It is well known that the accumulation of seminal vesicle secretion (SVS), which contains a group of proteins that constitute the major protein components of seminal plasma, becomes prominent in the postpubertal period. Finding the structure and function of these proteins has been attempted, to understand their roles in seminal vesicle physiology and their effects on the gamete activity. The rodent has proved to be a good experimental animal for this purpose and some of the protein components in rat and mouse SVS have been purified and identified [1–5].

We have shown that the antiserum obtained from the autoimmunization of male mice or the isoimmunization of female mice with mouse SVS is immunoreactive to an androgen-stimulated 19 kDa glycoprotein in mouse SVS [5,6]. This glycoprotein, which we designate tentatively as seminal vesicle autoantigen (SVA), has a core protein consisting of 131 amino acid residues. However, its primary structure, which shows no significant similarity to protein sequences collected in the data bank [5], give no clues to its function despite the demonstration that it can be complexed with Zn^{2+} [7]. In these circumstances, finding its action target is a prerequisite for a better understanding of its role in reproduction. The present study was performed with this aim. We found that (1) SVA is a novel phospholipid-binding protein secreted from the luminal epithelium of the primary and secondary folds in seminal vesicle, and (2) SVA binds sperm membrane phospholipids to suppress sperm motility.

EXPERIMENTAL

Materials

Phosphatidylcholine (PtdCho), lysophosphatidylcholine, phosphatidylethanolamine (PtdEtn) and lysophosphatidylethanolamine from egg yolk; phosphatidic acid and lysophosphatidic acid from egg yolk lectin; PtdIns from pig liver, phosphatidylserine (PtdSer) and sphingomyelin (SPM) from bovine brain; and fatty-acid-free BSA, biotin *N*-hydroxysuccinimide, 5-bromo-4-chloroindol-3-yl phosphate, FITC, Nitro Blue Tetrazolium and poly(vinyl alcohol) (soluble in cold water) were purchased from Sigma (St. Louis, MO, U.S.A.). Pronase, biotin-conjugated anti-rabbit IgG, FITC-conjugated anti-rabbit IgG and alkaline-phosphatase-conjugated streptavidin were purchased from Boehringer-Mannheim (Mannheim, Germany). Poly(isobutyl methacrylate) was obtained from Aldrich (Milwaukee, WI, U.S.A). Na^{125}I was from Amersham Corp. (Arlington Heights, IL, U.S.A.). Paraffin (Paraplast, m.p. 56–57 °C) was from Curtin Matheson Scientific Co. (Houston, TX, U.S.A.). Vetabond™ reagent was from Vector Laboratories (Burlingame, CA, U.S.A.). PD-10 columns and Percoll were obtained from Pharmacia (Uppsala, Sweden). Aluminium-backed silica-gel TLC plates

Abbreviations used: BSP, bovine seminal plasma; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; SPM, sphingomyelin; SVA, seminal vesicle autoantigen; SVS, seminal vesicle secretion; TBS, Tris-buffered saline; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.

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and Whatman GF/C glass microfibre filters were purchased from Whatman (Maidstone, Kent, U.K.). Iodobeads were from Pierce (Rockford, IL, U.S.A.). All chemicals were of reagent grade.

Preparation of SVA and its derivatives

Outbred ICR mice were purchased from Charles River Laboratories (Wilmington, MA, U.S.A.). They were bred in the animal centre at the College of Medicine (National Taiwan University, Taipei, Taiwan). Animals were treated in accordance with the institutional guidelines for the care and use of experimental animals. They were kept under controlled lighting (14 h light/10 h dark) at 21–22 °C with a supply of water and NIH 31 laboratory mouse chow *ad libitum*. SVA I and SVA II were purified from mouse SVS as described previously [5]; they were collected from mature male mice (8–12 weeks old). SVA I was used throughout the study.

In the preparations of SVA derivatives, a PD-10 column was used for the separation of the protein conjugate from free ligands. Before use, the column was presaturated with 0.5% BSA in a buffer and washed with the same buffer until no BSA appeared in the eluate, as measured by A_{280}.

FITC-labelled SVA was prepared by a modified method of Maxfield et al. [8]. A solution containing 40 μM SVA and 400 μM FITC in 50 mM borate buffer, pH 9.2, was gently stirred for 2 h in the dark at room temperature. Ethanolamine was added at a final concentration of 100 mM to stop the reaction. The reaction mixture was passed through a PD-10 column, that had been pre-equilibrated with the same buffer. The protein peak was collected, dialysed against distilled water, freeze-dried and stored at −70 °C before use.

Biotinylated SVA was prepared as described previously [9]. To 490 μl of 20.4 μM SVA in 0.1 M $NaHCO_3$ containing 0.2 M NaCl at pH 8.3, 10 μl of 20 mM biotin *N*-hydroxysuccinimide in dimethylformamide was added; the molar ratio of reagent to protein was 20. The mixture was stirred gently at room temperature for 2 h. This reaction was stopped by the addition of 50 μl of ethanolamine. The biotinylated SVA was separated through a PD-10 column in the same way as the preparation of FITC-labelled SVA mentioned above.

We followed a modified method of Markwell [10] to prepare ^{125}I-SVA. In brief, 60 μl of $Na^{125}I$ (1.0 mCi) from a commercial source was mixed with 77 μg of SVA in 50 μl of 100 mM Tris/HCl, pH 7.4, in the presence of Iodobeads. The radiolabelled protein was separated from free $Na^{125}I$ through a PD-10 column pre-equilibrated with 100 mM Tris/HCl, pH 7.4. ^{125}I-SVA, which gave a specific radioactivity of 70–100 μCi/μg of protein, showed a single band and was indistinguishable from its parent protein by migration on SDS/PAGE.

Preparation of spermatozoa and assay of sperm motility

In accordance with a previous method [11], the pH of modified Tyrode's buffer, which consisted of 100 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 25 mM $NaHCO_3$, 5.6 mM glucose, 0.5 mM sodium pyruvate, 25 mM sodium lactate, 1.0 mg/ml poly(vinyl alcohol), 100 i.u./ml penicillin and 100 μg/ml streptomycin, was adjusted to 7.3–7.4 by aeration with humidified air/CO_2 (19:1) in an incubator at 37 °C for 48 h before use. Poly(vinyl alcohol) was added to serve as a sperm protectant [12]. Adult male mice (12–16 weeks) were killed by cervical dislocation. The epididymides were removed and immersed in the medium. After they had been carefully dissected away from the connective tissues, spermatozoa were extruded from the distal portion of the tissues at 37 °C for 10 min. The cells were gently filtered through two layers of nylon gauze, layered on top of a linear gradient of 20–80% (v/v) Percoll and centrifuged at 275 ***g*** at room temperature for 30 min [13,14]. Three distinct cell layers were formed. The lowest layer, which contained more than 95% viable cells with progressive motility, was diluted with 3 vol. of modified Tyrode's solution and centrifuged at 60 ***g*** for 10 min at room temperature. The cell pellets were resuspended and centrifuged twice more in the same way. The cell pellets were resuspended in a specified medium for further study. Sperm motility and cell morphology were observed in modified Tyrode's buffer under a phase-contrast microscope (IX-70; Olympus, Tokyo, Japan).

The parameters associated with the cell motility were determined by computer-assisted sperm assay with a sperm motility analyser (IVOS version 10; Hamilton-Thorne Research, Beverly, MA, U.S.A.). A 7.0 μl sample was placed in a 10 μm deep Makler chamber at 37 °C. The analyser was set as follows: negative phase-contrast optics and recording at 60 frames/s, minimum contrast 40, minimum cell size four pixels, low size gate 0.2, high size gate 1.5, low intensity gate 0.5, high intensity gate 1.5, non-motile head size 29, non-motile head intensity 76, medium average path velocity (VAP) 50 μm/s, low VAP 7.0 μm/s, slow motile cells yes, and threshold STR greater than 80%. Fifteen fields were assessed for each sample.

Preparation of sperm lipid

The total lipids of spermatozoa were extracted by a modified method of Folch et al. [15]. The cells were suspended in 1.0 ml of 0.05 M KCl, sonicated in a Branson ultrasonic water bath (Model B-52) for 5 min at room temperature, and extracted twice with 3.0 ml of chloroform/methanol (2:1, v/v). The lipid extract was dried by flushing with N_2 and redissolved in a suitable organic solvent for TLC. Meanwhile the sperm lipid was dissolved in ethanol and injected through a 26-gauge needle into PBS (ethanol/PBS 1:20, v/v). The suspension was repeatedly forced through the needle, sonicated in the Branson ultrasonic water bath for 1 h at room temperature, centrifuged at 18000 ***g*** and used immediately in the study of the inhibitory effect on SVA–sperm binding.

Histological studies

Antiserum against SVA was raised in New Zealand White rabbits and used in the immunohistochemical study. Tissues were fixed in freshly prepared Bouin's solution [0.2% picric acid/2% (v/v) formaldehyde in PBS] overnight, dehydrated in ethanol, infiltrated and embedded in paraffin. Each tissue section (4 μm) was mounted on a slide that had been precoated with Vetabond reagent. Sections were dried at 45 °C, paraffin was removed in xylene and the sections were rehydrated through a gradient from alcohol to distilled water. The rehydrated sections were placed in a saturated lead thiocyanate solution and heated with the use of the method of von Wasielewski et al. [16]. After the slides had been cooled at room temperature for 15 min, they were rinsed in distilled water and PBS each for 5 min. The slides were immersed in a blocking solution [5% (v/v) non-fat skimmed milk in PBS] in a moisture chamber at 25 °C for 1 h and washed with PBS containing 0.05% (v/v) Tween 20 (PBST) four times, each for 15 min. The seminal vesicle sections on slides were incubated with the SVA-induced antiserum diluted 1:500 in the blocking solution. After the slides had been gently agitated in four changes of PBST for 15 min each, the antibodies against SVA were immunodetected with alkaline-phosphatase-conjugated anti-rabbit IgG diluted 1:1000 in the blocking solution. The enzyme activity staining was conducted in the presence of 0.033% Nitro

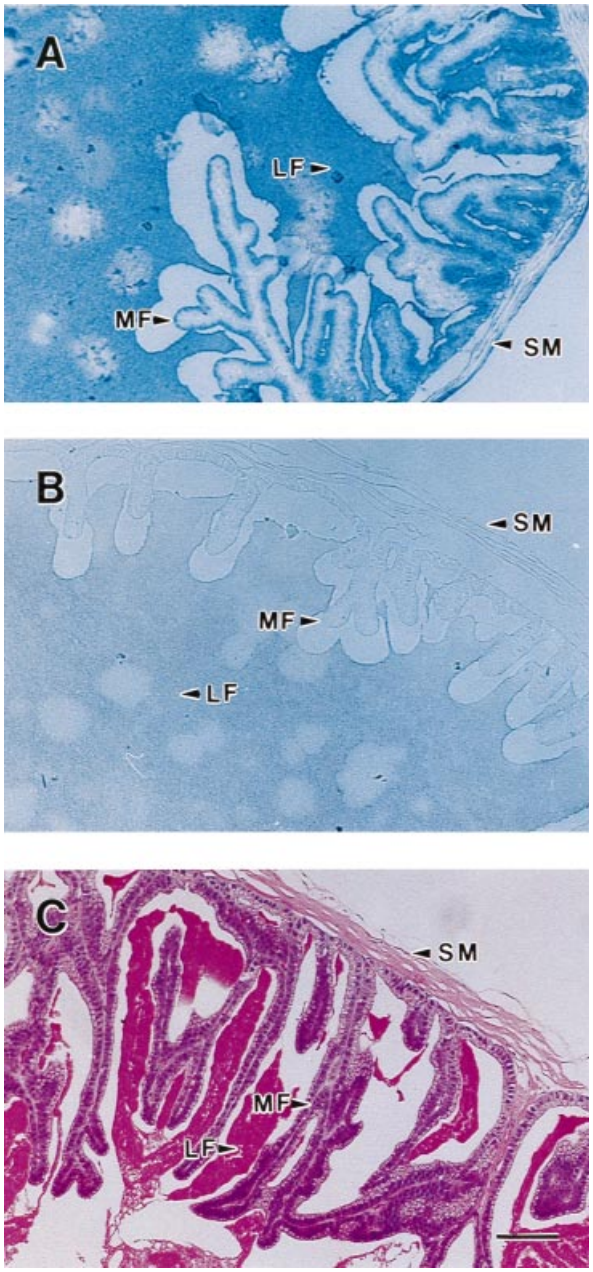

Figure 1 Histochemical staining patterns of SVA on sections of seminal vesicles of adult mice

(**A**) Tissue slices were histochemically stained for SVA with the antiserum against SVA and alkaline-phosphatase-conjugated anti-rabbit IgG. SVA was immunolocalized to the epithelium of primary and secondary mucosa folds. (**B**) The specimens were stained as in (**A**) except that the SVA antiserum was replaced by normal serum. (**C**) The specimens were stained with haematoxylin and eosin to reveal their morphology. Abbreviations: MF, mucosa fold; SM, smooth muscle; LF, luminal fluid. Scale bar, 100 μm.

Blue Tetrazolium and 0.0165% 5-bromo-4-chloroindol-3-yl phosphate in 100 mM Tris/HCl containing 100 mM NaCl and 5 mM $MgCl_2$ at pH 9.0 for 15 min at room temperature. The sections were washed with three changes of PBS for 15 min each.

Freshly prepared spermatozoa preincubated in the blocking solution (5×10^6 cells/ml) for 1 h were incubated with both 0.2 μM FITC-labelled SVA in the dark for 1 h and 0.7 μM biotinylated SVA alone or in the presence of a 5000-fold excess of unlabelled ligand for 1 h at room temperature. At the end of incubation, the cells were centrifuged and the cell pellets were washed with PBS to remove the unbound ligands. The cells treated with the biotinylated SVA were further incubated with alkaline-phosphatase-conjugated streptavidin diluted 1:1000 in the blocking solution for 1 h. The cells were agitated gently in three changes of PBS for 10 min each. The cells associated with the biotinylated SVA that bound to alkaline-phosphatase-conjugated streptavidin were then subjected to enzyme activity staining. After treatment, the cells were smeared on a slide; all slides were rinsed with PBS.

The specimens on a slide were covered with 50% (v/v) glycerol in PBS and photographed with a microscope equipped with epifluorescence (AH3-RFCA; Olympus).

Binding assay

Sperm lipid (1 ml) dispersed in PBS containing 1.3 μM SVA was stirred gently at room temperature for 1 h. Spermatozoa that had been pretreated in the blocking solution for 1 h were incubated in the mixture of SVA and sperm lipid for 1 h at room temperature. The cells were collected and immunoreacted with the SVA-induced antiserum and alkaline-phosphatase-conjugated anti-rabbit IgG as described in the Histological study section. The activity of the immobilized phosphatase on the sperm surface, which reflected the extent of SVA–sperm binding, was assayed in a solution containing 0.1% *p*-nitrophenyl phosphate, 100 mM glycine, 1.0 mM $MgCl_2$ and 1.0 mM $ZnCl_2$, pH 10.2, for 10 min at room temperature. The absorbance of the solution separated from the cells was measured at 405 nm and represented the extent of hydrolysis of the substrate *p*-nitrophenyl phosphate.

Spermatozoa (10^6 cells/ml) were incubated with 80 nM ^{125}I-SVA in 100 mM Tris/HCl at pH 7.4 in the presence of 0–770 μM SVA at 25 °C for 1 h. The cells were collected on a Whatman GF/C glass microfibre filter by rapid filtration at a pressure of 50.66 kPa (0.5 atm). In accordance with the method described previously [17,18], the filter had been blocked with 5% (v/v) non-fat skimmed milk in the buffer for 30 min and washed with the same ice-cold buffer before use. This minimized the background binding of ^{125}I-SVA to the filters. The filter was washed with six changes of 0.2 ml of the same ice-cold buffer and air-dried on a filter paper. The radioactivity of the filter was counted with a γ-counter. The non-specific binding of ^{125}I-SVA to cells was determined by the addition of a 10000-fold excess of unlabelled ligand. The specific binding of ^{125}I-SVA to cells was obtained by subtraction of the non-specific binding from the total binding. Each assay was conducted in triplicate and the IC_{50} was determined.

Binding of ^{125}I-SVA to phospholipids on a TLC plate generally followed the method of Desnoyers and Manjunath [19]. Sperm lipids and purified lipids were chromatographed on aluminium-backed silica-gel TLC plates in chloroform/methanol/water (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 for 1 h at room temperature in PBS containing 5% (v/v) non-fat skimmed milk. Plates were overlaid with ^{125}I-SVA (100000 c.p.m./ml) in the blocking buffer (100 μl/cm^2), incubated for 90 min at 25 °C, washed five times, each for 1–2 min, with cold PBS, dried and exposed to X-ray film for 18–36 h. The lipids on triplicate plates were also detected by spraying the plates with phosphomolybdic acid solution [20] or Dragendorff reagent [21].

The fluorescence intensity of SVA in Tris-buffered saline (TBS), expressed in arbitrary units, was measured at room

temperature with a Hitachi F-4000 fluorescence spectrophotometer. Both the excitation and the emission slit widths were 10 nm. Raman emission due to the scattering of solvent was minimized by adjusting the intensity scale. It took no more than 5 min to scan a spectrum, avoiding protein denaturation. The fluorescence intensity at wavelength λ_2 (nm) when the fluorophore was excited at wavelength λ_1 (nm) is denoted by $F_{\lambda 1}^{\lambda 2}$. A modified Scatchard plot [22] was constructed to analyse the fluorescence data of a complex formed by SVA and PtdCho:

$$|\Delta F|/[\mathrm{L}]_{\mathrm{free}} = F_{\infty}/K_{\mathrm{d}} - |\Delta F|/K_{\mathrm{d}} \quad (1)$$

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

RESULTS

Secretion of SVA and its binding sites on sperm surface

Among the non-sexual organs and the reproductive tracts of adult mice, our previous results of Northern and Western blot analyses demonstrated that SVA and its RNA message are present only in the seminal vesicle and that gene expression in this accessory sexual gland is androgen-dependent [5]. Figure 1 shows the immunohistochemical staining patterns of SVA in the

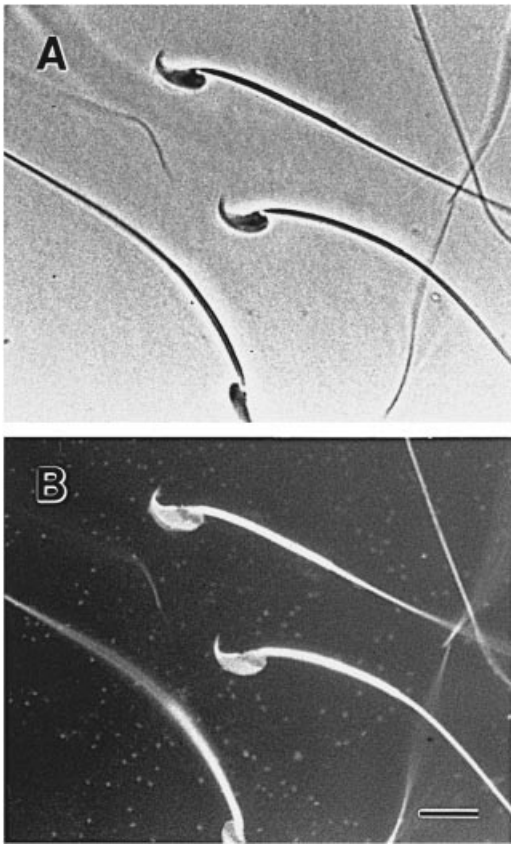

Figure 2 Demonstration of the SVA-binding zone on the spermatozoa

Fresh cells were incubated in blocking buffer in the presence of 0.2 μM FITC-labelled SVA in the dark at room temperature for 1 h. The cells were smeared on a slide and observed by phase-contrast microscopy (**A**) or fluorescence microscopy (**B**). Scale bar, 10 μm.

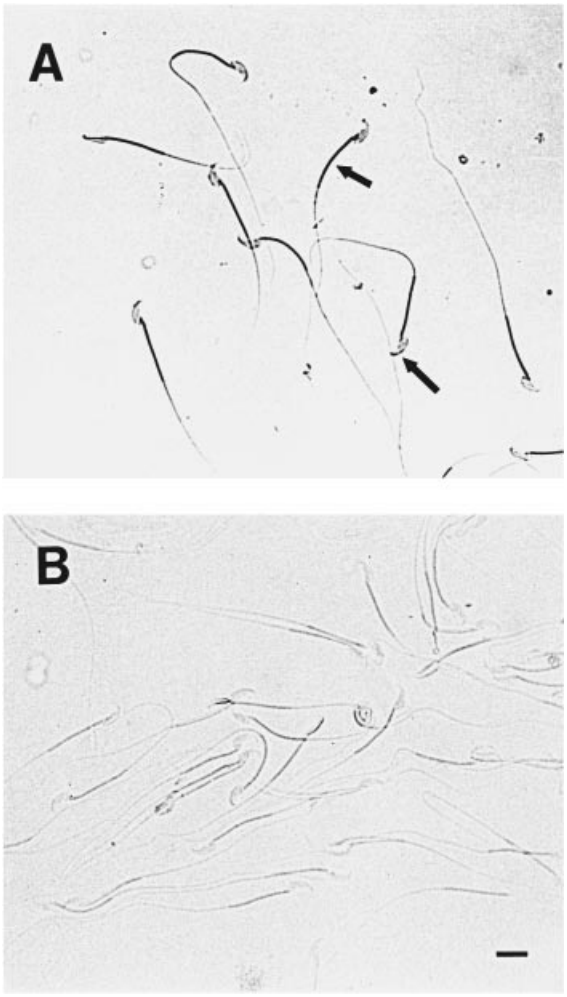

Figure 3 Specific binding of SVA to spermatozoa

Spermatozoa were incubated with 0.7 μM biotinylated SVA alone (**A**) or in the presence of a 5000-fold molar excess of SVA (**B**). Biotinylated SVA on the cell surface was detected by activity staining for alkaline-phosphatase-conjugated streptavidin, which binds to the biotin moiety. The dark deposits on the cell surface resulting from the enzymic reaction are indicated by arrows. Scale bar, 10 μm.

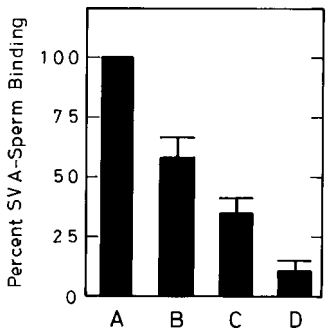

Figure 4 Inhibition of SVA–sperm binding by the dispersed lipid prepared from total sperm lipid

Spermatozoa (5×10^6 cells/ml) in PBS, pH 7.4, were incubated with 1.3 μM SVA and the dispersed sperm lipid prepared from 2.5×10^8 cells (column B), 10^9 cells (column C) or 2.5×10^9 cells (column D). The extent of SVA binding to sperm was assayed as described in the text. Results are percentages of SVA–sperm binding measured in the absence of sperm lipid from the cell incubation (column A) and are expressed as means $\pm$ S.D.

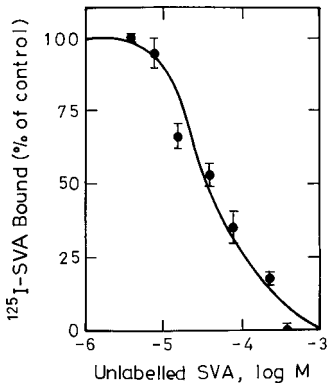

Figure 5 Inhibition of the binding of ^{125}I-SVA to spermatozoa by SVA

Spermatozoa in 100 mM Tris/HCl containing 5% (v/v) non-fat skimmed milk at pH 7.4 (10^6 cells/ml) were incubated for 1 h in the presence of 80 nM ^{125}I-SVA and 0–770 μM SVA at 25 °C. Radioactivity associated with the cells was measured (see the text for details). Results are expressed as percentages of the counts measured in the absence of unlabelled SVA. Points are means ± S.D. for three determinations.

tissue slices of mouse seminal vesicles. The protein was mainly immunolocalized to the luminal epithelium of the primary and the secondary mucosa folds. The staining intensity was much lower in the layer of smooth muscle. The strong immunochemical staining intensity of SVA in the lumen supports the idea that SVA accumulates in the lumen as a result of its secretion from the luminal epithelium. The amount of SVA in SVS was determined as 0.9% (w/v) by an ELISA method.

No fluorescence appeared on the epididymal spermatozoa after they had been immunoreacted successively with the SVA antiserum and FITC-conjugated anti-rabbit IgG, manifesting the lack of SVA on the cell surface. When spermatozoa were incubated with 0.2 μM FITC-labelled SVA in the blocking solution at 25 °C for 1 h, the fluorescein fluorescence was visible around the entire cell surface (Figure 2). We replaced FITC-labelled SVA with biotinylated SVA (0.7 μM) in the cell incubation: the cytochemical staining for the biotinylated ligand was prominent around the anterior region of the acrosome and the midpiece region but a lower staining intensity was seen around the posterior region of the acrosome and the tail region (Figure 3A). The biotinylated SVA was barely stained on the cells by addition of a 5000-fold molar excess of SVA to the cell incubation (compare Figures 3A and 3B). Taken together, the results indicate that mouse spermatozoa have SVA-binding sites that cover the entire cell surface. Cells pretreated with pronase (1.0 mg/ml) in PBS at 37 °C for 1 h, heat at 100 °C for 10 min, or exposure to 100 mM HCl (pH 1.0) at 25 °C for 1 h still retained their stainability with biotinylated SVA (results not shown). Apparently the SVA-binding sites were not destroyed after these pretreatments. Furthermore, the binding of SVA to the sperm surface could be greatly suppressed by the presence of sperm lipid extract dispersed in the cell incubation. Figure 4 shows that as the quantity of dispersed sperm lipid in the cell incubation increased, the extent of SVA binding to sperm decreased.

^{125}I-SVA was used for the assay of SVA–sperm binding. Figure 5 shows the data from one representative determination. The radiolabelled SVA bound to the cell surface was completely inhibitable by unlabelled SVA with an IC_{50} of $(3.89 \pm 0.65) \times 10^{-5}$ M^{-1}. Having resolved a SDS extract of epididymal spermatozoa

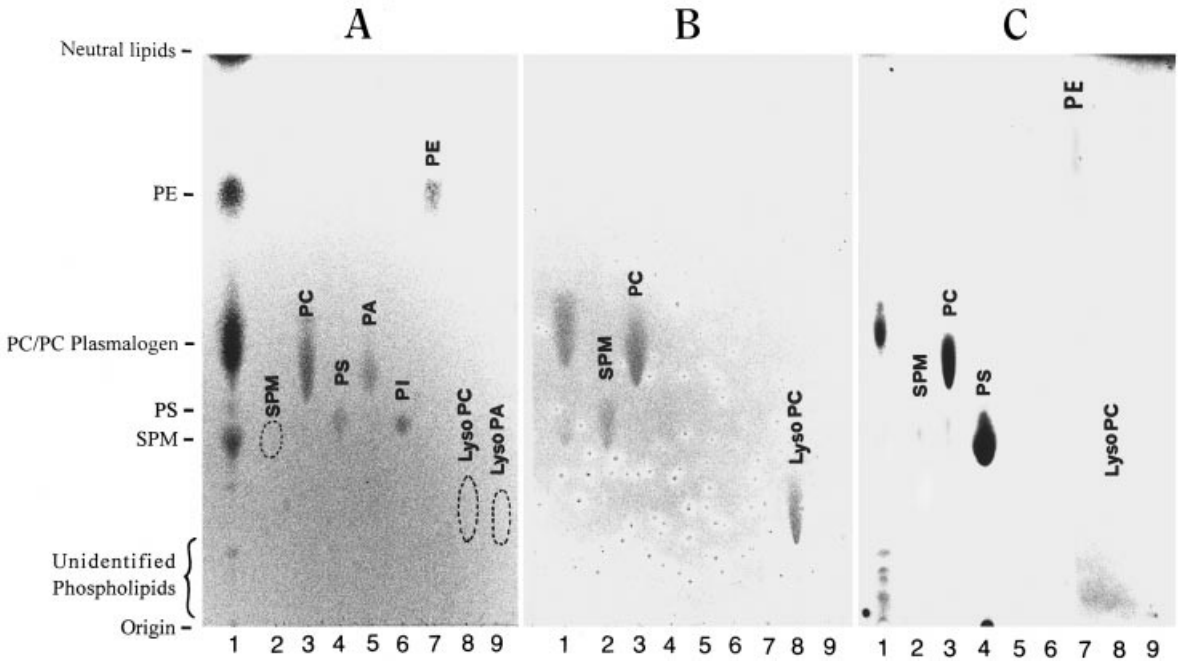

Figure 6 Binding of ^{125}I-SVA to phospholipids separated by TLC

Lipids extracted from 2.5×10^7 spermatozoa (lane 1) and 30 μg of each of the purified phospholipids (lanes 2–9) were spotted and chromatographed on silica-gel TLC plates in chloroform/methanol/water (65:25:4, by vol.). (**A**) Phospholipids detected with phosphomolybdic acid spray. (**B**) Choline-containing phospholipids detected with Dragendorff reagent spray. (**C**) Autoradiogram for the binding of radiolabelled SVA to separated phospholipids obtained after a TLC-overlay binding technique described in the text. Abbreviations: lyso PA, lysophosphatidic acid; lyso PC, lysophosphatidylcholine; PA, phosphatidic acid; PC, PtdCho; PE, PtdEtn; PI, PtdIns; PS, PtdSer.

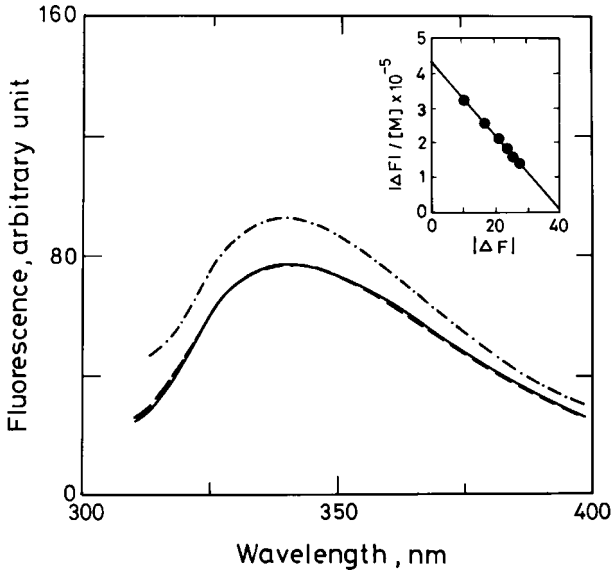

Figure 7 Effect of PtdCho on the fluorescence of SVA

The emission spectra were scanned with an excitation wavelength of 295 nm. The protein concentration was 1.6 μM in TBS, pH 7.4. Solid line, protein alone; broken line, 5% (v/v) ethanol in the protein solution; dot-dashed line, 40.0 μM PtdCho and 0.5% (v/v) ethanol in the protein solution. The inset shows a modified Scatchard plot of a fit of the fluorescence data from the addition of PtdCho to the protein solution by means of eqn. (1). The correlation coefficient of linear regression fitting was calculated as more than 0.98.

by SDS/PAGE, we failed to detect any binding of the separated protein components to ^{125}I-SVA by ligand blot analysis.

Characterization of SVA–lipid binding

The lipid extract of epididymal spermatozoa and purified phospholipids were chromatographed on silica gel-coated aluminium plates. Lipids on the plate were detected with phosphomolybdic acid spray, which detects phospholipid (Figure 6A), or with Dragendorff reagent, which specifically detects choline phospholipid (Figure 6B). As reported previously [19], the mouse sperm phospholipids were well separated in the developing solvent employed except that PtdCho and PtdCho plasmalogens migrated together. Sperm phospholipids were resolved into five major components and several minor components (lane 1 of Figures 6A and 6B). On the basis of the R_F values of purified lipids and the nature of lipid staining, the minor components remained unidentified and the main components were identified as neutral lipid, PtdEtn, PtdCho/PtdCho plasmalogen, PtdSer and SPM, with PtdSer in a relatively small amount. This accords with the phospholipid composition of spermatozoa reported previously [23]. The results of a TLC-overlay binding assay showed that ^{125}I-SVA gave a strong binding to purified PtdCho and PtdSer and a weak binding to purified SPM, lysophosphatidylcholine and PtdEtn but did not interact with phosphatidic acid, PtdIns or lysophosphatidic acid. Among the sperm lipids, ^{125}I-SVA showed strong binding to either PtdCho or the unidentified minor phospholipids, weak binding to SPM and neutral lipids, but did not interact with PtdEtn or PtdSer (lane 1 of Figure 6C). The amount of sperm PtdSer on the TLC plate might have been insufficient for the binding assay so that the radioactivity due to its binding with ^{125}I-SVA was not detectable.

SVA contains nine tyrosine residues and two tryptophan residues; the latter are Trp^{2} and Trp^{82}. Figure 7 displays the

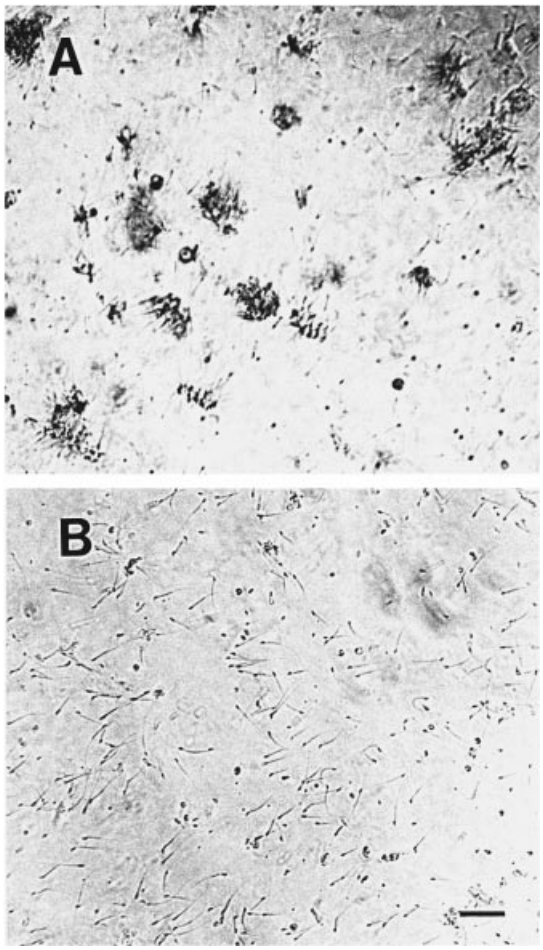

Figure 8 Spermatozoal morphology after cell incubation with SVA or BSA

Spermatozoa in modified Tyrode's buffer were incubated for 80 min in the presence of 0.3% BSA (**A**) or 0.3% SVA (**B**) at 37 °C. Cell morphology was photographed by phase-contrast microscopy. Scale bar, 100 μm.

emission of 1.6 μM SVA under various conditions. Excitation was at 295 nm to excite the fluorescence of the two tryptophan residues. The protein in TBS exhibits a peak at 345 nm (Figure 7, solid line), suggesting that the two tryptophan residues are on the protein surface but are restricted into a configuration that differs from that of free tryptophan, which gives a peak at 352 nm in aqueous solution. The presence of 5.0% (v/v) ethanol in the protein solution did not change the protein fluorescence in either spectral profile or emission intensity (compare the solid and broken lines in Figure 7), indicating a lack of effect of the organic solvent in the protein solution on the tryptophan status. In contrast, the addition of PtdCho dissolved in ethanol to final concentrations of 40 μM PtdCho and 0.5% (v/v) ethanol in the protein solution considerably enhanced the protein fluorescence intensity but the emission peak did not shift (compare the dot–dashed and solid lines in Figure 7). Apparently, PtdCho interacted with SVA to change the protein's fluorescence. Therefore PtdCho–SVA binding was probed by the ability of PtdCho to perturb the protein fluorescence. We fitted the F_{295}^{345} values, obtained by titrating 1.6 μM protein solution with PtdCho, to eqn. (1). As shown in the inset of Figure 7, the modified Scatchard plot is linear, supporting that there is a single type of

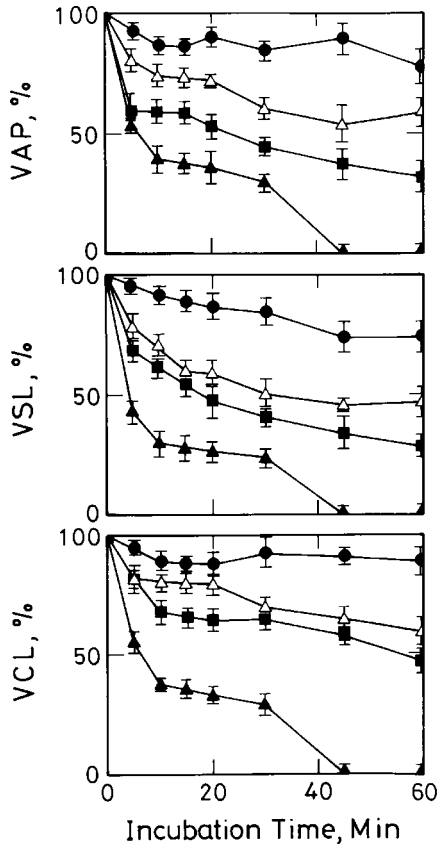

Figure 9 Analysis of sperm motility under the influence of SVA and BSA

Mouse sperm in modified Tyrode's buffer at pH 7.4 were incubated for 0–60 min in the presence of 0.3% BSA (●), 0.3% SVA (▲) or both together (■) at 37 °C. The parameters associated with the cell motility such as VAP, VSL and VCL were measured by computer-assisted sperm assay as described in the text. The results of each parameter determined at each specified incubation are expressed as percentages of control cell motility (△) measured at zero time incubation. Points are means ± S.D. for 10 determinations.

PtdCho-binding site on the protein molecule. The apparent K_d of PtdCho–SVA binding was estimated as $(9.10 \pm 0.02) \times 10^{-5}$ M^{-1}, which is compatible with the IC_{50} determined from SVA–sperm binding.

Suppression of sperm motility by SVA

Most spermatozoa freshly retrieved from mouse caudal epididymis in modified Tyrode's buffer retained their motility with tail beating even after incubation for 120 min at 37 °C. The presence of 0.3 % BSA in the cell incubation hyperactivated the cells with an apparent increase in both motility and the amplitude of their tail beats. Most of the BSA-treated cells stuck headfirst to the edge of an incubation well. When observed under a phase-contrast microscope, they appeared in clusters with the motile tails upwards and the head-to-head 'agglutination' downwards (Figure 8A). In contrast, sperm became immotile in the presence of 0.3 % SVA in the cell incubation. Each of the SVA-treated cells was quiescent at a fixed position, with no tail beating (Figure 8B). To examine how sperm motility was affected, we compared VAP, straight-line velocity (VSL) and curvilinear velocity (VCL) of spermatozoa incubated at 37 °C in the presence of 0.3 % BSA and/or 0.3 % SVA for 0–60 min. Relative to the motility of control cells, the value of each parameters was enhanced by BSA but was markedly suppressed by SVA at any incubation time (compare ●, △ and ▲ in Figure 9). VAP, VSL and VCL of sperm were not detectable after incubation for more than 40 min in the presence of SVA alone. When the cells were incubated with BSA and SVA together, each value of the three parameters was higher than that with SVA alone but was lower than that with BSA alone at any incubation time (compare ●, ■ and ▲ in Figure 9), showing that BSA was able to counteract the effect of SVA, and vice versa, with regard to sperm motility.

DISCUSSION

In the seminal vesicle, SVA is secreted predominantly from the luminal epithelium of mucosa folds to the lumen. Apparently, the effect of SVA–sperm binding on sperm activity should take place after ejaculation. Cytochemical observations (Figures 2 and 3) together with the SVA–sperm binding assay (Figure 5) supports the notion that the sperm surface has SVA-binding sites that cover the entire cell surface. However, the strength of SVA–sperm binding is rather weak compared with that of ordinary ligand–receptor binding. Because the binding sites are resistant to protease digestion or acid treatment and are heat stable, they might not be protein in nature. The demonstration that the dispersed sperm lipid was able to inhibit SVA–sperm binding (Figure 4) supports the notion that the SVA-binding sites are lipid in nature. In fact, ^{125}I-SVA failed to bind the protein components in an SDS extract of epididymal spermatozoa. ^{125}I-SVA blotting of the membrane phospholipids of spermatozoa on TLC plates (Figure 6C) suggests that PtdCho/PtdCho plasmalogen and SPM might constitute the major SVA-binding sites on the cell surface. These phosphocholine-containing lipids together make up more than 70 % of the total lipid in the plasma membrane of mouse spermatozoa [23]. The ability of SVA to bind to choline phospholipid was also confirmed by ligand blotting of purified lipids on the TLC plate (Figure 6C) and the analysis of the PtdCho-perturbed SVA fluorescence (Figure 7).

The phospholipid-binding proteins in the reproductive tract have been studied. Several bovine seminal plasma (BSP) proteins, which are the major secretory products of the seminal vesicle, have been purified and identified [24,25]. They belong to a family of closely related acidic proteins, designated BSP-A1, BSP-A2, BSP-A3 and BSP-30 kDa. It has been suggested that they have some role in the membrane modification of spermatozoa that occurs during capacitation and/or acrosome reaction through the interaction with the membrane phospholipids. In comparison with the specificity of SVA–phospholipid binding, BSP proteins bind strongly to the choline-containing phospholipids such as PtdCho, SPM and lysophosphatidylcholine; however, BSP-A1, A2 and A3 do not interact with phosphatidic acid, PtdEtn, PtdIns or PtdSer, and BSP-30 kDa protein shows weak binding to these phospholipids without a choline headgroup. Besides the difference in specificity between SVA–lipid and BSP–lipid binding, the primary structure of SVA shows no significant similarity to the protein sequences of BSP proteins. Furthermore, SVA is not related to any other phospholipid-binding proteins such as perforin [26], phosphocholine-binding protein [27], factor V [28], factor VIII [29,30], factors IX and X [31], p65 [32], pulmonary surfactant protein [33], C-reactive proteins [34], apolipoproteins A-I, A-II and A-IV [35] and lipid transfer proteins [36–38]. Therefore SVA represents a novel phospholipid-binding protein.

After ejaculation, the secretions of the male accessory sexual glands of mammals constitute the main portion of seminal fluids, which serve primarily as a vehicle. It is well known that some of its components stimulate the activity and metabolism of the

spermatozoa, and others provide the necessary energy. As stated by Barros [39], the fertile condition of spermatozoa is not a terminal condition but rather a transient one. After coitus of the rodent, the semen coagulates to form a copulation plug that fills the vagina and the ejaculated spermatozoa can be forced through the cervix into the uterus. They must spend a definite period in the female tracts to undergo some modification before they acquire the ability to fertilize the egg. However, cell modifications far from the oviduct would cause the cells to become infertile. Although the molecular events responsible for the cell modifications are not clearly understood, it is believed to be a multistep process and involves modification at the level of the sperm plasma membrane. Our results indicate that sperm motility can be hindered after ejaculation as the SVA molecules attach to the spermatozoal surface by specific interaction with membrane phospholipids containing a phosphocholine head group. This inhibitory effect might prevent spermatozoal maturation at any time earlier than the sperm–egg encounter. When spermatozoa are propelled to reach their destination by the orgasmal contraction of the female tracts, removal of the SVA effect is necessary if they are to resume their swimming movement, which is important not only to keep the cells in suspension and thus facilitate their transport but also to help individual cells through the coats that enclose the egg. In view of the observation that BSA counteracts the SVA effect, we suspect that the SVA effect might be removed during sperm passage in the uterine lumen, where the concentration of serum albumin is higher than in the other reproductive tracts. Further study is needed to clarify this point and to understand whether the SVA effect is associated with the incapacitation of spermatozoa in the prevention of a premature acrosome reaction.

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