Binding of seminalplasmin to the plasma and acrosomal membranes of bovine spermatozoa

Fluorescence studies on the changes in the lipid-phase fluidity

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Received 2 December 1985

The binding of seminalplasmin, a protein secreted by the accessory sex glands of bull, to the plasma and outer acrosomal membrane of bovine spermatozoa was studied using three different fluorescent probes. 8-Anilino-1-naphthalenesulfonate fluorescence, pyrene excimer fluorescence and diphenylhexatriene fluorescence polarisation studies indicate that seminalplasmin binds to the spermatozoal membranes, and leads to an increase in the fluidity of both the plasma and the acrosomal membranes. Calcium was found to have no influence on the interaction of seminalplasmin with the spermatozoal membranes. These results suggest that protein(s) present in the seminal plasma could interact with spermatozoal membranes and increase their fluidity.

Seminalplasmin *(Bovine spermatozoal membrane)* *Membrane fluidity* *Protein-membrane interaction*

1. INTRODUCTION

Seminalplasmin (SPLN), an antimicrobial protein isolated from bovine semen [1], inhibits the growth of both bacteria and yeast by inhibiting the synthesis of RNA [1–7]. This protein is secreted only by the accessory sex glands of bull, indicating that it interacts with spermatozoa for the first time only at the time of ejaculation [8]. Immunofluorescence studies indicated that SPLN coats the surface of ejaculated spermatozoa [5]. Binding of proteins to the surface of spermatozoa may influence capacitation, acrosome reaction and fertilising ability. The acrosome reaction is an obligatory membrane-fusion event which occurs prior to fertilisation during which the sperm plasma membrane in the head region, overlying the acrosome, selectively fuses with the outer acrosomal membrane to form vesicles [9]. The mechanism of the acrosome reaction is not clearly understood. The binding of seminal plasma proteins to spermatozoa in a variety of mammals [10–13] has been reported, however, very little is known as to whether these proteins modify the fluidity of membranes, a change known to precede the acrosome reaction [14–16]. Here, the interaction of SPLN with bovine spermatozoal plasma membrane (SPM) and acrosomal membrane (SAM) was studied using 3 fluorescent probes, viz. 8-anilino-1-naphthalenesulfonate (ANS), pyrene and diphenylhexatriene (DPH). The results indicate that SPLN, a constituent of seminal plasma, interacts with both SPM and SAM and causes an increase in the fluidity of these membranes.

2. MATERIALS AND METHODS

The plasma and outer acrosomal membrane fractions of bull spermatozoa were isolated from a 5 g pellet of spermatozoa by the method of Zahler and Doak [17] as described [18,19]. SPLN was purified from bovine semen [1,20]. Protein was determined by the method of Lowry et al. [21].

Fluorescence measurements were recorded on a Hitachi 650-S fluorescence spectrophotometer, operated in the ratio mode, with 4 nm excitation

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

and emission bandpass. A 2 mM aqueous solution of recrystallised ANS served as a stock. ANS was excited at 370 nm. Pyrene was incorporated into SPM and SAM by rapidly mixing a stock solution of 2 mM in methanol with the membrane, such that the alcohol concentration did not exceed 1%. The excitation wavelength used was 333 nm; the monomer emission was observed at 372 and 392 nm and the excimer emission was at 470 nm. For DPH polarization experiments, a stock solution of 2 mM in tetrahydrofuran was added directly to the membranes such that the final concentration of the probe was 1 μM, mixed thoroughly for 10 min on a vortex shaker and then incubated at 37°C for 30 min. Measurements were made using λ (excitation) = 355 nm and λ (emission) = 430 nm. All solutions for fluorescence studies were prepared in 10 mM Tris-HCl buffer (pH 7.4).

3. RESULTS AND DISCUSSION

The binding of the anionic fluorescent probe ANS is determined both by membrane surface charge [22] and the degree of lipid disorder [23]. The emission spectra of ANS in the presence of SPM and SAM and the influence of SPLN on these membranes are shown in fig.1. Binding of ANS to the membranes is accompanied by large intensity enhancements, with SPM showing a greater intensity change than SAM (fig.1A,B). The emission peak of ANS was also blue-shifted from 510 to 470 and 480 nm in the presence of SPM and SAM, respectively. Addition of SPLN to SPM and SAM in the presence of ANS caused a further increase in the fluorescence emission intensity (fig.1A,B) and the increase in intensity was dependent on the amount of SPLN added, finally levelling off. SPLN by itself altered the fluorescence intensity of ANS minimally and shifted the emission maximum to 490 nm.

The emission peaks of membrane-bound ANS were seen to be blue-shifted in the presence of SPLN by 5 and 10 nm in the case of SPM and SAM, respectively. Scatchard analyses of the data obtained by fluorescence titrations of membranes in the presence or absence of SPLN indicated that SPM binds more probe as compared to SAM and, in the presence of SPLN, both show an increase in their ability to bind to the probe (table 1). Since ANS binding reflects charge and packing effects – more disordered membranes accommodating greater amount of probe [23] – it appears that SPLN increases the disorder in the membranes.

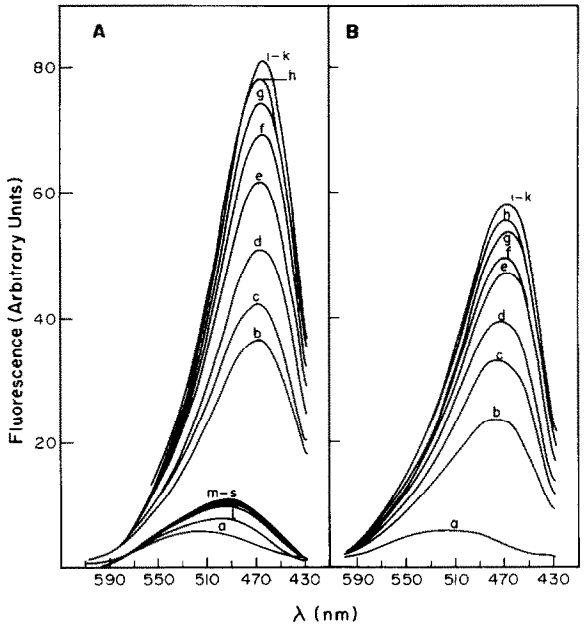

Fig.1. Fluorescence spectra of ANS in the presence of plasma and acrosomal membranes of bovine spermatozoa. λ (excitation), 370 nm; ANS concentration, 25 μM; protein concentration, 100 μg/ml. (A) a, free ANS; b, a + 100 μg SPM; c–k, successive addition of 10 μg SPLN to b; l–s, successive addition of 10 μg SPLN to a. (B) a, free ANS; b, a + 100 μg SAM; c–k as in A.

To confirm the above observation, the interaction of SPLN with sperm membranes was monitored using pyrene. The fluorescence emission intensity of the excimer peak of pyrene

Table 1

Binding of ANS to plasma and acrosomal membranes of bovine spermatozoa in the presence of seminalplasmin

Membrane	Seminalplasmin (μg/ml)	ANS	
		No. of binding sites (n)	K_d (μmol/mg protein)
SPM	–	118 ± 5	0.8 ± 0.02
SPM	40	144 ± 12	1.08 ± 0.07
SAM	–	88 ± 9.5	1.0 ± 0.3
SAM	40	119 ± 10	1.1 ± 0.25

The concentration of membrane protein was 100 μg/ml

(470 nm) can be conveniently used to monitor membrane fluidity, since the formation of the excimer is related to the lateral mobility of pyrene molecules in the lipid phase [24,25]. The emission spectra of pyrene incorporated into SPM are shown in fig.2A; fig.2B shows the excimer/monomer (E/M) emission intensity ratio of pyrene in SPM and SAM, and the influence of SPLN on this ratio. The excimer formation is more prominent in SPM as compared to SAM indicating that SPM is more fluid. This confirms our earlier observation that SPM has a more fluid lipid phase than SAM [19]. SPLN alone does not increase the excimer intensity or the E/M ratio of pyrene (fig.2B) but, when added to SPM or SAM, it increases the excimer intensity and thus the E/M ratio in a concentration-dependent manner (fig.2A,B). Hence it appears that SPLN increases the fluidity of both SPM and SAM.

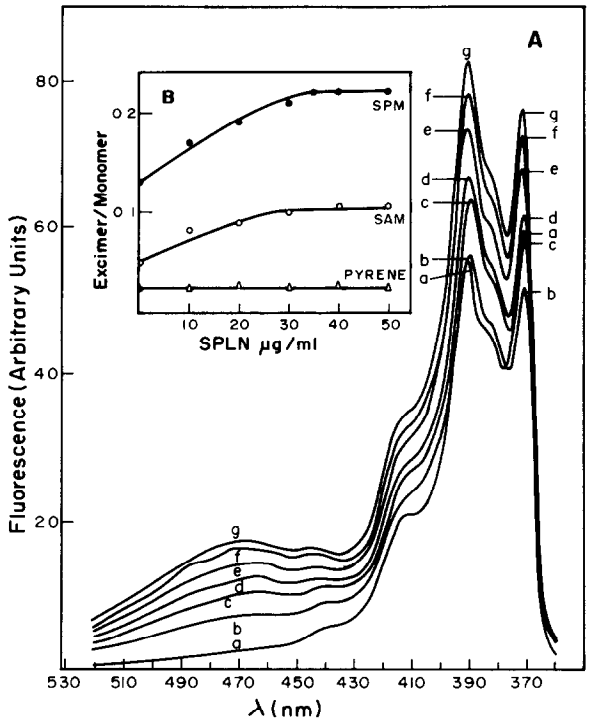

Fig.2. (A) Uncorrected fluorescence emission spectra of pyrene (4 μM) incorporated into plasma membranes of bovine spermatozoa. Protein concentration, 50 μg/ml; λ (excitation), 333 nm. a, free pyrene; b, a + 100 μg SPM; c–g, successive addition of 10 μg SPLN to b. (B) Excimer/monomer intensity ratio of pyrene (4 μM) incorporated into plasma and acrosomal membranes of bovine spermatozoa, as a function of seminalplasmin concentration. Membrane protein concentration, 50 μg/ml.

Ca^{2+} has been implicated in membrane changes that occur during the acrosome reaction [26–29]. The role of Ca^{2+} in modulating membrane fluidity, inducing phase separation in the lipid bilayer and facilitating membrane fusion has also been stressed [30]. Hence, the interaction of SPLN with SPM and SAM was studied in the presence of Ca^{2+}. It was observed that Ca^{2+} up to 10 mM did not have any effect on the excimer fluorescence of pyrene in the membranes. Addition of SPLN to SPM and SAM in the presence or absence of 10 mM Ca^{2+} also brought about the same change in fluidity. Addition of Ca^{2+} (2–10 mM) to membranes incubated with SPLN did not increase the fluidity further. Ca^{2+} is needed for the acrosome reaction, but its role in the fusion of plasma and acrosomal membranes is not clearly understood [28,29].

Fluorescence polarization measurement using DPH is one of the most efficient methods of determining microviscosity (η) of membranes [31]. The results in table 2 indicate that the microviscosity of SAM is higher than that of SPM, in good agreement with the results with pyrine and the observed higher cholesterol content of SPM as compared to SAM [19]. A significant decrease in the microviscosity of SPM and SAM was also noted upon addition of SPLN to the membranes. Thus, the experiments with pyrene and DPH indicate that SPLN binds to spermatozoal membranes and increases their fluidity. Certain proteins like cytochrome *c* interact with phospholipid bilayers and lower the phase transition temperature – an effect

Table 2

Microviscosity of plasma and acrosomal membranes of bovine spermatozoa in the presence of seminalplasmin determined using DPH

Seminalplasmin (μg/ml)	Microviscosity (P)	
	SPM	SAM
0	2.77 ± 0.18	4.10 ± 0.03
10	2.60 ± 0.13	3.80 ± 0.10
20	2.55 ± 0.12	3.30 ± 0.20
30	2.27 ± 0.01	3.02 ± 0.06
40	2.05 ± 0.14	2.69 ± 0.12

The concentration of membrane protein was 100 μg/ml, and that of DPH, 1 μM. λ (excitation) = 355 nm:λ (emission) = 430 nm

that indicates increase in fluidity [32]. BSA is also known to interact with rat spermatozoa and to increase the fluidity of the membranes [14–16]. Earlier reports have indicated that proteins secreted by the accessory sex glands of rat [13], rabbit [10], mouse [11] and bull [12] bind to spermatozoa on ejaculation; however, changes in membrane fluidity were not investigated in these reports.

The present results confirm our earlier observations that the lipid phase of SPM is more fluid than the lipids of SAM [19]. SAM is responsible for sequestering the hydrolytic enzymes of the acrosome until the occurrence of the acrosome reaction; hence a more rigid membrane would be justified. However, both these membranes are involved in the formation of mixed vesicles of SPM and SAM during the acrosome reaction [9]. While the molecular events responsible for this membrane fusion are not clear, Davis [14] observed that prior to the acrosome reaction, during capacitation, rat spermatozoa incubated in the presence of BSA showed a decrease in cholesterol/phospholipid ratio, making the membranes more fluid; this change could promote fusion of SPM and SAM [14,15]. The observed increase in the fluidity of SPM and SAM on interaction with SPLN is consistent with the model proposed by Davis [14] for molecular changes preceding acrosome reaction wherein it is suggested that both the plasma and outer acrosomal membrane of spermatozoa undergo increase in membrane fluidity prior to intracellular increase in Ca^{2+} and membrane fusion.

ACKNOWLEDGEMENTS

I thank Drs P.M. Bhargava, D. Balasubramanian, D. Chatterji and Bina Chandani for useful discussions.

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