Major proteins of bovine seminal plasma inhibit phospholipase A₂

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We have recently shown that the major proteins of bovine seminal plasma, namely BSP-A1, BSP-A2, BSP-A3 and BSP-30-kDa (collectively called BSP proteins) bind to spermatozoa and that the binding sites on the plasma membrane of spermatozoa are choline phospholipids. In view of the fact that these phospholipids are substrates for phospholipase A₂ (PLA₂), a key enzyme in sperm capacitation and the acrosome reaction, the effect of BSP proteins on this enzyme activity was investigated. Since these BSP proteins are ubiquitous, the effect on pig pancreatic PLA₂ was also studied. In contrast with control proteins, when preincubated with phosphatidylcholine as substrate, all BSP proteins inhibited both pancreatic and sperm PLA₂ activity in a dose-dependent manner and in the presence of 1–6 μ M BSP protein the enzyme activity was completely abolished. When phosphatidylethanolamine was used as substrate, only pancreatic PLA₂ was inhibited. On the other hand,

when the BSP proteins were preincubated with the enzyme followed by addition of substrate, a biphasic effect was observed; there was stimulation of enzyme activity below 1.3 μ M BSP followed by an inhibition above this concentration. The inhibitory activity was trypsin-sensitive but heat-resistant. The effect of co-incubation of heparin, which is implicated in sperm capacitation and which also interacts with BSP proteins, was studied. Heparin (10 μ M) had no effect on the PLA₂ inhibitory activity exhibited by all BSP proteins. The PLA₂ inhibitory effect exhibited by BSP proteins was abolished with excess substrate. The BSP proteins were adsorbed on PLA₂-agarose and could be affinity cross-linked to the enzyme, indicating a direct interaction of enzyme with the inhibitor. These results suggest that these BSP proteins modulate PLA₂ activity and therefore, phospholipid metabolism.

INTRODUCTION

Bovine seminal plasma (BSP) contains a family of closely related acidic proteins which we have designated BSP-A1, BSP-A2, BSP-A3 and BSP-30-kDa (collectively called BSP proteins) (Manjunath, 1984; Manjunath and Sairam, 1987; Manjunath et al., 1987). These proteins are secretory products of the seminal vesicles (Manjunath et al., 1987) and they constitute the major proteins of BSP. The biochemical characteristics and structure of these proteins have been studied in detail (Manjunath and Sairam, 1987; Manjunath et al., 1988). BSP-A1, -A2 and -A3 have molecular masses of 15–16.5 kDa whereas the BSP-30-kDa protein has a molecular mass of 28–30 kDa. All members of this family of proteins are glycosylated with the exception of BSP-A3. BSP-A1 and -A2 have identical amino acid sequences but differ in the extent of glycosylation. Together, these two proteins are also called PDC-109 (Esch et al., 1983). On the other hand, both BSP-A3 and -30-kDa proteins have amino acid compositions that are different from -A1/-A2. The determination of their amino acid sequences show that BSP-A1/-A2 and -A3 proteins are structurally organized into two tandemly arranged repeating units of 38–41 amino acids (Esch et al., 1983; Seidah et al., 1987) that are similar to type-II structures present in the gelatin-binding domain of fibronectin (Baker 1985; Seidah et al., 1987), human plasma factor XII (McMullen and Fujikawa, 1985), insulin-like growth factor II (IGF-II) or cation-independent mannose-6-phosphate receptor (Morgan et al., 1987; Lobel et al., 1988; Oshima et al., 1988) and type-IV collagenase (Collier et al., 1988). Type-II structures present in these BSP proteins also interact with several macromolecules; different genetic types of collagens (types I, II, IV and V), fibrinogen (Manjunath et al.,

1988), heparin (Chandonnet et al., 1990), calmodulin (Manjunath et al., 1993), apolipoprotein A-I (apoA-I) and apoA-I associated with high-density lipoproteins (HDL; Manjunath et al., 1989). In view of these binding properties it would appear that these BSP proteins are multifunctional.

Our previous results have shown that the BSP proteins bind to spermatozoa and other cell types (Manjunath et al., 1988, 1994). Recently, we identified the binding sites on cell membranes to be those phospholipids that contain the choline head group (Desnoyers and Manjunath, 1992). Based on this phospholipid-binding activity, as well as their interaction with HDL, we have proposed that the BSP proteins play an important role in sperm membrane lipid modification that occurs during capacitation.

In the present study we show that BSP proteins are potent inhibitors of sperm and pancreatic PLA₂. The inhibition appears to be due to a direct interaction of BSP proteins with PLA₂ although a substrate depletion mechanism cannot be ruled out. The results further support our suggestion that the BSP proteins are indeed involved in sperm capacitation and acrosome reaction.

MATERIALS AND METHODS

Materials

Lactoperoxidase, heparin, calmodulin, BSA and PLA₂ (600 units/mg) from pig pancreas were purchased from Sigma (St. Louis, MO, U.S.A.). Phosphatidylcholine (PC) (L- α -1-palmitoyl-2-[¹⁴C]linoleoyl) (specific radioactivity 55.6 mCi/mmol), phosphatidylethanolamine (PE), (L- α -1-palmitoyl-2-[¹⁴C]arachidonoyl) (specific radioactivity 52.6 mCi/mmol) and scintillation fluid (P-989) were obtained from New England Nuclear (Mississauga, Ont., Canada). Na¹²⁵I and PE (L- α -1-

Abbreviations used: BSP, bovine seminal plasma; HDL, high-density lipoprotein; apo, apolipoprotein; PLA₂, phospholipase A₂; BS³, Bis(sulphosuccinimidyl) suberate; TBS, Tris-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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palmitoyl-2-[^{14}C]linoleoyl) (specific radioactivity 55.6 mCi/mmol) were obtained from Amersham (Oakville, Ont., Canada). Trypsin from bovine pancreas was purchased from Boehringer-Mannheim (West Germany). *Bis*(sulphosuccinimidyl) suberate (BS^3) was purchased from Pierce Laboratories (Rockford, IL, U.S.A.). Affi-Gel 15 and electrophoresis reagents were from Bio-Rad. Aluminium-backed silica-gel t.l.c. plates (250 μm thick) were from Whatman Ltd. (Maidstone, Kent, U.K.). All other chemicals used were of analytical grade and were purchased from commercial suppliers.

Bovine semen was obtained from the Veterinary Medical School of the University of Montreal (St-Hyacinthe, Québec, Canada). BSP-A1, -A2, -A3 and -30-kDa proteins of bovine seminal plasma were purified by gelatin-agarose affinity chromatography (Manjunath et al., 1987). In view of the fact that BSP-A1 and -A2 have identical amino acid sequences but contain different amounts of carbohydrate these molecular forms will be considered as a single chemical unit and will be referred to as BSP-A1/-A2.

Iodination of PLA_2

Pig pancreas PLA_2 was labelled with ^{125}I using the lactoperoxidase method as described earlier (Manjunath and Sairam, 1982). The labelled PLA_2 was separated from free iodine by gel filtration on a 20 ml column of Sephadex G-25 equilibrated with 50 mM sodium phosphate, pH 7.4, containing 150 mM NaCl, 0.1% BSA and 0.2% sodium azide. The specific radioactivity of radiolabelled PLA_2 was in the range of 60–80 $\mu\text{Ci}/\mu\text{g}$.

Human sperm PLA_2 preparation

PLA_2 from human ejaculated spermatozoa was prepared by the method of Guérette et al. (1988). In brief, spermatozoa were centrifuged at 940 g for 10 min. The pellet was resuspended with 10 ml of Tris-buffered saline (TBS; 50 mM Tris/HCl, 150 mM NaCl, pH 8.2) and recentrifuged for 10 min at 3800 g . The washing was repeated twice and the pellet (spermatozoa) was resuspended in 50 mM Tris/HCl buffer, pH 8.2, containing 4 mM deoxycholate. The suspension was sonicated for 2 min in a Branson ultrasonic water bath (Model 5200) and centrifuged (10000 g , 10 min). The supernatant containing PLA_2 activity was frozen at -20°C in aliquots of 100 μl and was found to be stable in this state for at least several months. Frozen aliquots were thawed when required and diluted to give solutions of 1 mg/ml proteins and used for assay. Using PE as substrate at pH 8.2, the enzyme had a specific activity of 4 nmol/min per mg of protein.

Determination of PLA_2 activity

The activity of pig PLA_2 was determined by the release of ^{14}C -labelled fatty acid from radiolabelled PC or PE using the method described by Miele et al. (1988) with minor modifications. A typical reaction mixture contained 50 mM Tris/HCl (pH 9), 100 mM NaCl, 1 mM CaCl_2 and 1 mM sodium deoxycholate, 3.6 μM radioactive phospholipids (0.36 nmol per assay) and 10 ng of pig PLA_2 in a total volume of 100 μl . The reaction was started by the addition of the enzyme to the radioactive substrate. After incubation at 37°C for 10 min, the reaction was stopped by adding 200 μl of chloroform/methanol (2:1, v/v) and 50 μl of 4 M KCl. The reaction mixture was then vortexed and centrifuged (10000 g , 3 min) to separate the aqueous phase. The organic

phase containing radioactive free fatty acids and phospholipids was applied to aluminium-backed silica gel t.l.c. plates. The radioactive free fatty acids were separated by developing in the solvent system; petroleum ether/ethyl ether/acetic acid (85/15/1, by vol.). The iodine-stained bands co-migrating with authentic free fatty acid were scraped directly into vials, mixed with scintillation fluid and the radioactivity was determined in an LKB liquid-scintillation counter. The percentage conversion of substrate into free fatty acid was calculated as an index of enzyme activity. Blanks in the absence of enzyme were included in all experiments.

The activity of sperm PLA_2 was determined using PC or PE as substrate as described for the pancreatic enzyme except that the pH employed was 8.2. Our previous studies have shown that the sperm enzyme shows maximal activity at pH 8.2 and that PE is the preferred substrate (Guérette et al., 1988). The quantity of the enzyme used was 5 μl (1 mg/ml protein) and the incubation period was 3 min. Any other changes in the assay conditions are described under the respective figures.

To determine the PLA_2 inhibitory activity, the substrate was preincubated for 15 min with the putative inhibitors (BSP proteins) at 37°C and the reaction was initiated by adding the enzyme, PLA_2 . In some experiments, PLA_2 was preincubated for 15 min with the BSP proteins and the reaction was initiated by the addition of substrate. The enzyme activity was measured as described above and the percentage inhibition was calculated in relation to the control value (without inhibitors).

Digestion of BSP proteins by trypsin

Solutions of BSP proteins (1 mg/ml in 0.1 M NH_4HCO_3 containing 0.1 mM CaCl_2) were incubated at 37°C and 10 μl of trypsin (1 mg/ml) from bovine pancreas was added (enzyme/substrate ratio of 1:100, w/w). Aliquots of 100 μl were removed at designated time intervals and the trypsin was inactivated by immediately mixing with soya-bean trypsin inhibitor (10 $\times$) or by boiling the digest for 10 min. The samples were then lyophilized and assayed for pancreas PLA_2 inhibitory activity. In parallel experiments, protein solutions were also treated similarly without trypsin to provide the appropriate controls.

Coupling of PLA_2 to Affi-Gel 15

Coupling of pig PLA_2 to Affi-Gel 15 was carried out essentially as described for the coupling of BSP-A1/-A2 to Affi-Gel 15 (Manjunath et al., 1989). In brief, 5 ml of gel slurry (Affi-Gel 15) was transferred to a small Buchner funnel and washed with five bed volumes of cold (4°C) glass-distilled water. The moist gel cake was then transferred to a 15 ml plastic culture tube and 4.5 ml of pig PLA_2 solution (1 mg/ml dialysed against 0.1 M Mes buffer, pH 6.5) was added. The tube was kept shaking at 4°C on an end-over-end shaker for 18 h. Then 0.4 ml of 1 M ethanolamine/HCl (pH 8) was added to block any remaining active esters. After one more hour of shaking to complete the blocking reaction, the gel was transferred to a small column (2 ml bed volume) and washed with 8–10 bed volumes of 25 mM Tris/HCl, pH 7.4. Coupling efficiency was 95%, approximately equivalent to 2 mg of PLA_2 bound per ml of gel. Affi-gel was also reacted with ethanolamine as above in the absence of protein and this gel was used as a control in the binding experiments.

Affinity-cross linking

BSP-A1/-A22 proteins were incubated with ^{125}I - PLA_2 (50000 c.p.m.; 0.15 ng) in 45 μl of buffer (1 mM Tris/HCl,

pH 7.5) for 18 h at 4 °C. Samples were then treated at 4 °C with 5 μ l of BS³ (final concentration of 1 mM) for 10 min and the reaction was quenched by the addition of 3 μ l of 1 M NH₄HCO₃. Electrophoresis sample buffer was added to give a final concentration of 5 mM Tris/HCl, pH 6.8, 5% (w/v) SDS, 10% (w/v) glycerol and 10% (v/v) 2-mercaptoethanol. Samples were heated (100 °C for 10 min) and submitted to SDS/PAGE. Gels were stained with Coomassie Blue R-250, dried and exposed to X-ray films (RX, Fuji) for 2–5 days.

Analytical methods

SDS/PAGE in 15% (w/v) polyacrylamide gels was performed according to Laemmli (1970) using a Bio-Rad Protein II vertical slab gel electrophoresis apparatus. Proteins were stained with Coomassie Brilliant Blue R-250. The apparent molecular mass was determined using the low-molecular-mass calibration kit from Pharmacia (Canada). For calculations, molecular masses of 16 kDa for BSP-A1/-A2 and -A3, and 28 kDa for BSP-30-kDa protein were used. Protein concentrations were determined by the method of Bradford (1976) using a Bio-Rad dye-binding kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.) with BSA used as standard.

RESULTS

The assay conditions were first standardized with respect to ionic strength, calcium ion concentration, time and pH. Using this data we established the linearity of the reaction with respect to enzyme concentration. The activity of pig pancreatic PLA₂ was linear over a range of 5–20 ng per tube when assayed at pH 9 for 20 min with a PC concentration of 0.36 nmol/tube (3.6 μ M). The amount of pig enzyme selected for most experiments was 10 ng/tube and the incubation time was 10 min at 37 °C. Under these conditions less than 15% of substrate was hydrolysed. The activity of sperm PLA₂ was linear over the range of 2–15 μ l per tube (5 μ g of protein/ml) when assayed using PE as substrate at pH 8.2 for 10 min. The amount of sperm enzyme selected for most experiments was 5 μ l/tube and the incubation time was 3 min at 37 °C. Under these conditions the sperm enzyme hydrolysed less than 15% of the added substrate.

It should be noted that the pancreas and sperm PLA₂ activity results reported here were obtained using substrates dispersed in buffer containing deoxycholate. In our initial experiments we have used substrate prepared by sonication in incubation buffers containing no deoxycholate. Although PLA₂ hydrolysed substrates in deoxycholate micelles more rapidly than substrates in aqueous liposomes, the results obtained were similar (results not shown). An extensive comparison of enzyme activity using substrates prepared by these two methods was not done.

Effect of preincubation of BSP proteins with PC as substrate on pancreatic and sperm PLA₂

The effect of preincubation of (L- α -1-palmitoyl-2-[¹⁴C]linoleoyl)-PC with increasing concentrations of BSP proteins on pig pancreatic PLA₂ activity is shown in Figure 1(a). There was no change in PLA₂ activity up to 0.2 μ g of BSP proteins preincubated with substrate; however, the presence of BSP proteins in the reaction mixture above this concentration resulted in the inhibition of PLA₂ activity. This inhibition was dose dependent and with 3–10 μ g of BSP proteins ($\sim$ 2 μ M -A1/-A2; $\sim$ 4–6 μ M -3 and $\sim$ 1 μ M -30-kDa), there was a total inhibition of PLA₂

activity. The concentration of the protein required for 50% inhibition of enzyme activity (ID₅₀) was approx. 1.2–1.5 μ g (0.5–1 μ M) for all of the BSP proteins.

Sperm PLA₂ was also inhibited in a similar manner (Figure 1b) when BSP proteins were co-incubated with PC substrate before determination of PLA₂ activity. This inhibition was again dose-dependent and at approx. 6 μ g of BSP protein (4 μ M for -A1/-A2 and -A3, 2 μ M for -30-kDa) there was a total inhibition of sperm PLA₂ activity. ID₅₀s were approx. 1.5 μ g or 0.9 μ M for -A1/-A2 and -A3 proteins and approx. 2.5 μ g or 0.9 μ M for the -30-kDa protein. In contrast, preincubation of PC with several control proteins (cytochrome c, BSA, lysozyme and γ -globulin) under similar conditions had no significant effect on either pancreatic or sperm PLA₂ activity (results not shown).

Effect of preincubation of BSP proteins with PE substrate on pancreatic and sperm PLA₂

Inhibitory activity in the presence of BSP proteins using (L- α -1-palmitoyl-2-[¹⁴C]arachidonyl)-PE as substrate was determined to

Figure 1 Effect of preincubation of BSP proteins with PC substrate on pancreatic and sperm PLA₂

(a) Inhibition of pancreatic PLA₂. (L- α -1-palmitoyl-2-[¹⁴C]linoleoyl)-PC (0.36 nmol/tube) was preincubated with different concentrations of BSP proteins for 15 min at 37 °C and then 10 ng of pig pancreatic PLA₂ was added. After a 10 min incubation at 37 °C, the released radioactive fatty acids were separated by t.l.c. and assayed (details in the Materials and methods section). BSP-A1/-A2 (○), -A3 (●) and -30-kDa (△). (b) Inhibition of sperm PLA₂. Conditions were the same as above except that 5 μ l of sperm enzyme was added and the incubation time was 3 min. BSP-A1/-A2 (○), -A3 (●) and -30-kDa (△). Each value represents the mean of three experiments done in triplicate; S.D.s were less than 13% of the mean values.

Figure 2 Effect of preincubation of BSP proteins with PE substrate on pancreatic and sperm PLA₂

(a) Inhibition of pancreatic PLA₂. (L- α -1-palmitoyl-2-[¹⁴C]arachidonyl)-PE (0.36 nmol/tube) was preincubated with different concentrations of BSP proteins for 15 min at 37 °C and then 5 ng of pig pancreatic PLA₂ was added. After a 10 min incubation at 37 °C, the released radioactive fatty acids were separated by t.l.c. and quantified. (b) Inhibition of sperm PLA₂. Conditions were the same as above except 5 μ l of the sperm enzyme preparation was added and the incubation time was 3 min (details in the Materials and methods section). Key to symbols: $\circ$, BSP-A1/-A2; $\bullet$, -A3; $\triangle$, -30-kDa. Each value represents the average of three experiments done in duplicate; S.D.s were never higher than 12% of the mean values.

establish the head group specificity. The inhibition patterns for pancreatic and sperm PLA₂ were different (Figure 2). While the inhibition of pig pancreatic PLA₂ activity by BSP-30-kDa protein was similar (ID_{50} = 2.3 μ g or 0.8 μ M) to that found with PC as substrate, the inhibition by -A1/-A2 was less dramatic (ID_{50} = 8 μ g or 5 μ M; Figure 2a). On the contrary, BSP-A3 protein caused no significant inhibition. Furthermore, ~10 μ M of the BSP-30-kDa protein completely inhibited PLA₂ activity, whereas at twice this concentration of -A1/-A2 nearly 80% of the activity was inhibited. Similar results were obtained with a different fatty acid at the *sn*-2 position of the substrate, (L- α -1-palmitoyl-2-[¹⁴C]linoleoyl)-PE (results not shown).

In contrast with the sperm enzyme there was no significant inhibition of PLA₂ activity by BSP-A1/-A2 and -30-kDa proteins using 1-palmitoyl-2-[¹⁴C]arachidonyl-PE, whereas the -A3 protein showed approx. 30% inhibition (Figure 2b). Results were similar when 1-palmitoyl-2-[¹⁴C]linoleoyl-PE was used as substrate (results not shown).

Figure 3 Effect of preincubation of BSP proteins on pig PLA₂

(a) Pig PLA₂ (10 ng) was preincubated with different concentrations of BSP proteins for 15 min at 37 °C and the reaction was initiated by the addition of (L- α -1-palmitoyl-2-[¹⁴C]linoleoyl)-PC. After a 10 min incubation at the same temperature, the released radioactive fatty acids were assayed and PLA₂ activity was computed. (b) The assay was performed as in (a) except that the substrate used was (L- α -1-palmitoyl-2-[¹⁴C]linoleoyl)-PE and the amount of enzyme used was 5 ng. Key to symbols: $\circ$, BSP-A1/-A2; $\bullet$, -A3; $\triangle$, -30-kDa. The results shown are averages of triplicate assays carried out on three occasions; S.D.s were never higher than 15% of the mean values.

Effect of preincubation of BSP proteins with PLA₂

Preincubation of BSP proteins with substrate followed by the addition of PLA₂ enzyme shows dose-dependent inhibition (Figures 1 and 2). In contrast, preincubation of pig PLA₂ with BSP proteins followed by the addition of substrate resulted in a biphasic effect on the enzyme activity (Figure 3). There was stimulation of enzyme activity (1.5- to 2.5-fold) in the presence of 0.1–2 μ g (0.06–1.3 μ M) of BSP protein followed by a dose-dependent inhibition above 2 μ g of protein. BSP-30-kDa protein was more stimulatory (2.5-fold) than -A1/-A2 or -A3 protein (Figure 3a). Preincubation with enzyme also caused an increase in the ID_{50} s when compared with preincubation with substrate. For the BSP-A1/-A2, -A3 and -30-kDa proteins, the ID_{50} s were 2.5 μ g (1.5 μ M), 7 μ g (4.4 μ M) and 6 μ g (2.2 μ M) respectively. When 1-palmitoyl-2-[¹⁴C]arachidonyl-PE was used as substrate, the results obtained were different (Figure 3b). Thus maximum stimulation was seen with BSP-A1/-A2 (up to 2-fold) and a 30–40% stimulation was observed with the -A3 and -30-kDa proteins. Furthermore, the ID_{50} s for BSP-30-kDa and -A1/-A2

Table 1 Effect of heat treatment on the inhibitory activity exhibited by BSP proteins

Samples (100 µg) of different BSP proteins in 100 µl of 0.05 M Tris/HCl buffer, pH 8, in glass tubes (triplicates) were held in boiling water for 10 min. The pancreas PLA₂ inhibitory activity was then determined as described in the Materials and methods section using radiolabelled PC as substrate. Results shown are averages of triplicate assays carried out on two occasions; S.D.s were never higher than 7–10% of the mean values.

Protein	Concentration (µg/tube)	PLA ₂ activity (%)	
		Control	Heat-treated
BSP-A1/-A2	1.56	36.3	31.9
	6.25	0.9	3.4
	25.0	0.0	0.0
BSP-A3	1.56	28.3	32.8
	6.25	13.1	13.2
	25.0	0.1	0.9
BSP-30-kDa	1.56	38.5	36.3
	6.25	1.7	2.3
	25.0	0.0	0.4

Figure 4 Effect of trypsin on PLA₂ inhibitory activity caused by BSP proteins

BSP-A1/-A2 protein was treated with trypsin for 15 min (○), 30 min (□), or 2 h (■) and the pancreas PLA₂ activity in the presence of the trypsin-treated proteins was determined using PC as substrate and compared with untreated protein (●). Details are given in the Materials and methods section. Note that the results obtained with the BSP-A3 and -30-kDa protein were similar. The results shown are averages of triplicate assays carried out on three occasions; S.D.s were never higher than 10–15% of the mean values.

proteins were 6 µg (2.2 µM) and 28 µg (17.5 µM) respectively. It is interesting to note that there was no inhibition of enzyme activity with the BSP-A3 protein at the highest concentration (30 µg/assay or 18 µM) tested. Similar results were obtained when 1-palmitoyl-2-[¹⁴C]linoleoyl-PE was used (results not shown). No such stimulatory effect was observed with the sperm enzyme using PC or PE as substrates (results not shown).

Effect of heat treatment on PLA₂ inhibition

All three BSP proteins retained complete inhibitory activity even after boiling for 10 min in solution (0.05 mM Tris/HCl, pH 8),

Table 2 Effect of substrate concentration on PLA₂ inhibition by BSP proteins

(L-α-1-palmitoyl-2-[¹⁴C]linoleoyl)-PC (0.2–13 µM) was preincubated with BSP protein (1 µM) and 10 ng of pig pancreatic PLA₂ (a) or 2 µl of sperm enzyme (b) was added, and the enzyme activity was determined as described in the Materials and methods section. Percentage enzyme activity remaining was calculated in relation to control values obtained with pancreas or sperm enzyme incubated with increasing amounts of substrate in the absence of inhibitors (BSP proteins). The results shown are averages of triplicate assays carried out on three occasions; S.D.s were less than 12% of the mean values.

PLA ₂	Substrate (µM)	PLA ₂ activity (%)		
		BSP-A1/-A2	BSP-A3	BSP-30-kDa
(a) Pancreas	0.32	0	0	0
	0.63	6	10	0
	1.27	28	22	3
	2.53	54	48	44
	5.06	85	84	115
	10.13	92	113	132
(b) Sperm	0.20	0	0	0
	0.40	0	7	0
	0.80	0	9	4
	1.62	36	16	13
	3.24	51	27	21
	6.48	64	49	34
	12.96	78	62	61

indicating that the inhibitory activity exhibited by these BSP proteins is stable to heat treatment (Table 1).

Effect of trypsin on PLA₂ inhibitory activity caused by BSP proteins

Each BSP protein was treated with trypsin for different time intervals and the pancreas PLA₂ activity in the presence of trypsin-treated proteins was determined using PC as substrate. The PLA₂ inhibitory activity exhibited by the BSP-A1/-A2, BSP-A3 and BSP-30-kDa proteins was retained following a 15 min treatment with trypsin, but after treatment for longer periods the inhibitory activity gradually decreased and was completely abolished after 2 h (Figure 4).

Effect of co-incubation of heparin with BSP proteins on PLA₂ inhibitory activity

Heparin interacts with BSP proteins and this glycosaminoglycan has been implicated in sperm capacitation and the acrosome reaction. Therefore, we investigated the effect of co-incubation of heparin and BSP proteins on PLA₂ inhibitory activity. The presence of heparin (up to 10 µg per assay) in the reaction mixture had no significant effect on the sperm or pancreas PLA₂ inhibitory activity exhibited by all of the BSP proteins (results not shown).

Effect of substrate concentration on PLA₂ inhibition by BSP proteins

Increasing amounts of PC (0.2–13 µM) were preincubated with each BSP protein and PLA₂ activity was assayed. Up to approx. 0.6 µM substrate, there was complete inhibition of pig PLA₂ activity (Table 2). However, as the substrate concentration increased, the inhibitory effect caused by the BSP proteins gradually decreased and in the presence of approx. 10 µM substrate the inhibition was completely abolished. The results

Figure 5 Binding of BSP-A1/-A2 to PLA₂-agarose

Affinity chromatography was carried out at 4 °C. The affinity column (2 ml bed volume) was equilibrated with 25 mM Tris/HCl, pH 7.4, containing 1 mM CaCl₂ and 250 µg of BSP-A1/-A2 dissolved in 500 µl of the equilibrating buffer was applied. The column was stopped for about an hour to allow sufficient time for interaction of the BSP protein with the affinity matrix. The column was then washed extensively with Tris/HCl buffer and the bound protein was eluted with 25 mM Tris/glycine, pH 2.5, containing 1 M NaCl.

Figure 6 Affinity cross-linking of BSP-A1/-A2 to ¹²⁵I-PLA₂

(a) ¹²⁵I-PLA₂ (50 000 c.p.m.) was incubated with BSP-A1/-A2 (0 µg, lane 1; 0.1 µg, lane 2; 1 µg, lane 3; 5 µg, lane 4) for 18 h at 4 °C and cross-linked with BS³ as described in the Materials and methods section. Lane 5, ¹²⁵I-PLA₂ without any treatment. (b) ¹²⁵I-PLA₂ (50 000 c.p.m.) was incubated with BSP-A1/-A2 (200 ng) in the absence (lane 1) and in the presence of unlabelled PLA₂ (1 µg, lane 2; 10 µg, lane 3; 25 µg, lane 4) for 18 h at 4 °C and BS³ was added. The cross-linked proteins were examined by SDS/PAGE and autoradiography.

obtained with the sperm enzyme were similar (Table 2) to those of the pancreas enzyme, although the inhibition was not completely abolished.

Binding of BSP proteins to PLA₂-Affi-Gel

The possibility that these BSP proteins interact directly with PLA₂ was investigated first with ¹²⁵I-PLA₂ binding to BSP-A1/-A2-Affi-Gel 15. A maximum of 30 % of the radioactive enzyme was bound to the column. Similar results were obtained when

radiolabelled BSP protein binding to PLA₂-Affi-Gel 15 was studied. These results suggest either a weak binding or loss of interaction between the BSP proteins and PLA₂ upon iodination. On the contrary, when unlabelled BSP-A1/-A2 was passed through a column containing PLA₂-Affi-Gel beads most of the protein was bound to the column (Figure 5). Examination of the adsorbed fraction by h.p.l.c. (Manjunath and Sairam, 1987) and SDS/PAGE revealed the presence of BSP-A1/-A2.

Affinity cross-linking

Radiolabelled PLA₂ was incubated with purified BSP-A1/-A2, cross-linked with BS³ and analysed by SDS/PAGE under reducing conditions. After autoradiography of the dried gel, a band was visible at 32 kDa which corresponded to a cross-linked complex of BSP-A1/-A2 and ¹²⁵I-PLA₂ (Figure 6a, lanes 2–4). Higher-molecular-mass complexes, formed with one mol of ¹²⁵I-PLA₂ and two mol of BSP-A1/-A2, could also be visualized after longer exposure of the gels (results not shown). The specificity of ¹²⁵I-PLA₂ cross-linking to the 32 kDa complex was tested by including increasing concentrations of unlabelled PLA₂ in the binding assay. The covalent incorporation of ¹²⁵I-PLA₂ into the 32 kDa complex was reduced in a dose-dependent fashion (Figure 6b).

DISCUSSION

The phospholipases A₂ comprise a family of calcium ion-dependent enzymes that cleave the *sn*-2 bond of diacylphospholipids and alkyl- or alkenyl-acyl phospholipids, liberating free fatty acids and lysophospholipids (Dennis, 1983; Waite, 1987; Hazen et al., 1991). The presence of PLA₂ activity has been reported in human (Thakkar et al., 1984), mouse (Thakkar et al., 1983), guinea pig (Ono et al., 1982), hamster (Llanos et al., 1982; Riffo et al., 1992), ram (Hinkovska et al., 1987; Roldan and Mollinedo, 1991) and bull sperm (Weinman et al., 1986) as well as in the seminal plasma of human (Kunze et al., 1974; Kunze, 1981; Wurl and Kunze, 1985), bull and ram (Scott and Davidson, 1968; Rönkkö et al., 1991; Rönkkö, 1992). Using immunofluorescence studies, PLA₂ has been localized on the surface of ejaculated spermatozoa (Weinman et al., 1986; Rönkkö, 1992). PLA₂ appears to play an important role in the acrosome reaction, an exocytotic event (for reviews see Meizel, 1984; Langlais and Roberts, 1985; Yanagimachi, 1988). Recent studies with ram spermatozoa strongly indicate that PLA₂ activation is essential to bring about exocytosis of the sperm acrosome (Roldan and Fragio, 1993). The activation of PLA₂ generates detergent-like products, *cis*-unsaturated free fatty acids and lysophospholipids, that can act as membrane fusogens. Consequently, the regulation of sperm PLA₂ activity may be important in membrane alterations that occur during fertilization (capacitation and acrosome reaction).

BSP proteins (BSP-A1, -A2, -A3 and -30 kDa) are a family of major proteins (30–40 mg/ml) secreted by bovine seminal vesicles. These proteins bind to the surface of spermatozoa upon ejaculation by their interaction with the membrane choline phospholipids (Manjunath et al., 1988; Desnoyers and Manjunath, 1992; Manjunath et al., 1994). Since phospholipids are substrates for PLA₂, a key enzyme implicated in the acrosome reaction, the effect of BSP proteins on this enzyme activity was investigated. In addition, PLA₂ enzymes are probably present in all living cells, where they function in a number of regulatory processes including membrane composition remodelling, exocytosis and the biosynthesis of lipid mediators such as platelet-activating factor (PAF), prostaglandins, leukotrienes, and the

thromboxanes (Dennis, 1983; Waite, 1987). In view of the fact that BSP proteins are also ubiquitous in mammals (Manjunath et al., 1988; Leblond et al., 1993) we studied the effect of BSP proteins on the pancreatic enzyme.

Initially, we sought to use bovine sperm PLA₂ and attempted to purify PLA₂ using the method described for human sperm PLA₂ (Guérette et al., 1988). However, using either PC or PE as substrate under standard assay conditions, the activity of the enzyme in bovine sperm extract was almost undetectable. Presumably, this may be due to the presence of inhibitors of PLA₂ such as the BSP proteins (discussed later). Consequently, we used a partially purified human sperm enzyme to investigate the effect of BSP proteins. It should be noted that immunoreactive BSP proteins are present in human seminal fluid (Manjunath et al., 1988) and seminal fluids of several other mammals (Leblond et al., 1993).

Effect of BSP proteins on PLA₂

BSP proteins showed different effects on PLA₂ activity depending on whether substrate or enzyme was preincubated with the BSP proteins, the type of substrate (PC or PE) and enzyme source (pig pancreas or human sperm). For instance, preincubation of BSP proteins with PC as substrate inhibited both sperm and pancreatic PLA₂ activity. The inhibition was dose-dependent and was complete at 2–6 μ M BSP protein. On the other hand, if PLA₂ was preincubated with BSP proteins and then PC as substrate was added to the reaction mixture, a biphasic effect on the enzyme activity was observed: below 1.3 μ M of BSP proteins there was stimulation of the enzyme activity but above this concentration there was inhibition. The extent of this stimulation/inhibition was dependent on the type of BSP protein and substrate. BSP-30-kDa was more stimulatory (2.5-fold) when acting on PC as substrate whereas BSP-A1/-A2 was more stimulatory (2-fold) when acting on PE as substrate. Furthermore, BSP proteins were inhibitory only on the pancreatic PLA₂ when acting on PE as substrate. In addition, the extent of the inhibition of pancreatic enzyme with PE as substrate was dependent upon BSP proteins. ID₅₀s for the BSP-30-kDa protein were similar with PC and PE as substrates. BSP-A1/-A2 was nearly 10-fold more inhibitory when acting on PC than on PE, whereas -A3 was almost inactive when acting on PE as substrate. The reason for these discrepancies is not clear. It is possible that PLA₂ enzymes from different sources may exert different specificities with respect to substrates, or alternatively, the BSP proteins themselves may exert different specificities. This specificity may have some significance in the regulation of the sperm enzyme, for example, the BSP proteins may limit sperm PLA₂ acting on choline phospholipids and permit PLA₂ to hydrolyse other phospholipids to provide energy required for motility and/or other activities.

The inhibitory activity exerted by the BSP proteins was trypsin-sensitive, as treatment of BSP proteins with trypsin for 1 h completely abolished the inhibitory activity. However, the inhibitory activity exhibited by these BSP proteins was thermostable. Heparin, a glycosaminoglycan, has been shown to be a sperm-capacitation factor (First and Parrish, 1987; Miller and Ax, 1990). Heparin binds to the BSP proteins (Chandonnet et al., 1990); however, this interaction does not effect the PLA₂ inhibitory activity exhibited by BSP proteins.

Mechanism of PLA₂ inhibition by BSP proteins

The effect of BSP proteins was dependent on the enzyme source (sperm or pancreas), the substrate (PC or PE) as well as

experimental conditions (preincubation with substrate or enzyme). As described for the annexins, BSP proteins may exert inhibition of PLA₂ activity by substrate depletion or via a substrate coating mechanism (Davidson et al., 1987; Haigler et al., 1987) because BSP proteins show inhibition only at low substrate concentrations. As the concentration of the substrate was increased, the inhibition was gradually decreased and at high concentrations it was completely abolished. However, when PLA₂ was preincubated with BSP proteins, the enzyme activity was actually stimulated over 2-fold. Furthermore, BSP-A1/-A2 bound to a PLA₂-agarose affinity matrix. In addition, BSP-A1/-A2 could be cross-linked to ¹²⁵I-PLA₂. These results indicate a direct interaction of BSP proteins with the enzyme as described for the antiflammins (Miele et al., 1988). While our results favour a direct interaction of the BSP proteins with PLA₂, a substrate depletion mechanism cannot be ruled out. Thus it would appear that the exact mechanism of inhibition/stimulation of PLA₂ by these BSP proteins is complicated and further investigation is required to establish this more precisely.

Biological significance of inhibition/stimulation of PLA₂ by BSP proteins

In previous studies, we have shown that BSP proteins bind to the sperm surface (Manjunath et al., 1988, 1994) and the binding sites on the sperm membrane are choline phospholipids namely, PC, PC plasmalogens and sphingomyelins (Desnoyers and Manjunath, 1992). These choline phospholipids together constitute over 73 % of the total lipids in the bovine sperm membrane (Pursel and Graham, 1967; Clegg and Foote, 1973; Parks and Lynch, 1992). In another study, we have shown that BSP proteins interact with HDL (Manjunath et al., 1989). In view of these findings, we have suggested that the BSP proteins play an important role in the modification of the sperm plasma membrane that occur during capacitation (Manjunath et al., 1989, 1994; Desnoyers and Manjunath 1992). On the other hand, PLA₂ has been localized by immunoelectron microscopy studies on the plasma membrane (outer- and inner-leaflets) as well as in the acrosome and post-acrosomal substance of ejaculated bull sperm (Weinman et al., 1986). More recently, by an indirect immunofluorescence technique, it has been shown that PLA₂ is bound on the surface of ejaculated bull sperm but not on the epididymal sperm (Rönkkö, 1992). Interestingly, this immunofluorescence pattern overlaps with the immunolocalization of BSP proteins on the bull sperm (Manjunath et al., 1994), suggesting colocalization of BSP proteins and PLA₂. In view of this, along with the present findings, it is possible that the BSP proteins directly interact with membrane-bound PLA₂ enzyme. The membrane-bound PLA₂ may be stimulated or inhibited depending on the local concentration of BSP proteins. By regulating enzyme activity, BSP proteins may modulate the membrane lipid composition in sperm undergoing capacitation. Alternatively, since BSP proteins coat the surface of the spermatozoa upon ejaculation via a specific interaction with choline phospholipids they may limit the availability of these phospholipids for extracellular or membrane-bound PLA₂ action. In this context it should be noted that bovine seminal fluid, the extracellular milieu, contains PLA₂; however, using PC as substrate, the activity is almost undetectable (P. Manjunath and S. Soubeyrand, unpublished work). This is presumably because BSP proteins, which are inhibitors of PLA₂ activity, are present at a very high concentration (20–45 mg/ml) in seminal fluid. In our preliminary experiments we have found that the depletion of BSP proteins in seminal fluid by their specific adsorption on gelatin-agarose affinity matrix and DEAE-Sephadex columns

results in a more than 100-fold increase in PLA₂ activity. Whether the inhibition of this extracellular PLA₂ by BSP proteins has any physiological relevance warrants further investigation.

In a recent study, cloning of a membrane receptor for secretory PLA₂ has been reported (Lambeau et al., 1994). Interestingly, the extracellular domain of this receptor contains type-II structures which are similar to those domains present in BSP proteins. The exact role of the type-II domain in PLA₂ receptor is not clear at present.

In conclusion, these results show that BSP-A1, BSP-A2, BSP-A3 and BSP-30-kDa proteins of bovine seminal fluid influence PLA₂ activity. They inhibit both the sperm-bound and pancreatic enzyme when PC is used as the substrate. Since BSP proteins specifically interact with choline phospholipids on the surface of the spermatozoa it is likely that a physiological role for these proteins is associated with membrane phospholipids. For instance, BSP proteins may sequester choline phospholipids on the sperm surface and thereby block PLA₂ from acting on these phospholipids and prevent sperm from undergoing a premature acrosome reaction. Alternatively, BSP proteins may directly interact with the PLA₂ enzyme, alter its activity and thereby regulate those membrane modification events that are involved in capacitation.

This work was supported by the Natural Sciences and Engineering Research Council of Canada and the Special Programme of Research, Development and Research Training in Human Reproduction, World Health Organization.

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