Calmodulin-binding proteins in bovine semen

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An ^{125}I -labelled calmodulin gel overlay procedure was used to direct calmodulin-binding proteins in bovine spermatozoa and seminal plasma. Several calmodulin-binding proteins with molecular masses ranging from 12 to >200 kDa were detected in epididymal and ejaculated spermatozoa. Certain of these proteins exhibited preferential calmodulin-binding in the presence of Ca^{2+} , while others exhibited binding only in its absence. In seminal plasma, only two major proteins with molecular masses of 15 and 16 kDa showed a higher calmodulin-binding activity in the presence of Ca^{2+} , whereas several polypeptides in the range of 6–17 kDa bound higher amounts of radiolabelled calmodulin in the absence of Ca^{2+} . Our previous study has shown that a group of closely related major proteins, designated as BSP-A1, BSP-A2, BSP-A3 and BSP-30 kDa, isolated from bovine seminal plasma (BSP) have molecular masses in the range of 15–30 kDa. This prompted us to investigate whether these polypeptides from bovine seminal fluid interact with calmodulin. The results indicated that calmodulin binds to purified BSP-A1, -A2, -A3 and BSP-30 kDa proteins in the presence and absence of Ca^{2+} . Furthermore, many polypeptides of low molecular mass (6–14 kDa) in bovine seminal plasma that crossreact with these BSP proteins also show high calmodulin-binding activity, particularly in the absence of calcium. This was further demonstrated following the limited proteolysis of the BSP proteins. Several tryptic-peptides of BSP-A1/-A2 and BSP-30 kDa exhibited higher calmodulin-binding activity than the intact BSP proteins. In view of the key role of Ca^{2+} in triggering the acrosome reaction and the role of calmodulin in intracellular transport of calcium, it is suggested that BSP proteins are involved in sperm capacitation and the acrosome reaction.

Introduction

Calmodulin is an acidic protein of low molecular mass known for its ability to regulate many cellular processes in a calcium-dependent manner (for review, see Cheung, 1980; Means *et al.*, 1982). The presence of calmodulin has been demonstrated in spermatozoa isolated from a wide range of animals, including guinea-pig (Jones *et al.*, 1980), boar (Camatini *et al.*, 1986), bull (Feinberg *et al.*, 1981; Weinman *et al.*, 1986), hamster (Moore and Dedman, 1984) and rabbit (Jones *et al.*, 1980; Camatini *et al.*, 1991). Calmodulin constitutes about 0.1% of the total proteins in the sperm plasma membrane fraction in bovine species (Noland *et al.*, 1985). The distribution of calmodulin in sperm cells has been determined by immunofluorescence and, according to these reports, calmodulin is localized in the acrosomal, the postacrosomal and equatorial regions as well as at the neck and tip of the flagellum. This localization of calmodulin in the acrosomal region together with data showing the calcium dependence of the acrosome reaction has supported the hypothesis that calmodulin is involved in this exocytotic process (Jones *et al.*, 1980). Moreover, several calmodulin-binding proteins have been detected in hamster (Moore and Dedman,

1984), bull (Olson *et al.*, 1985) and boar (Peterson *et al.*, 1989) spermatozoa and it has been suggested that the regulation of calmodulin is mediated through its interaction with binding proteins. Leclerc *et al.* (1989, 1990) showed that ejaculated cryopreserved bull spermatozoa contain three major calmodulin-binding proteins with molecular masses of 28, 30 and 49 kDa. Heparin induced a decrease in the binding of these proteins to calmodulin and this finding was positively correlated to the fertilization rate. On the basis of these results, the authors suggest that sperm capacitation is associated with a decrease in the binding of calmodulin to sperm proteins of 28, 30 and 49 kDa. One of the approaches to gain further insight into elucidating the function of calmodulin in capacitation and the acrosome reaction is therefore to identify the calmodulin-binding proteins that are present in spermatozoa and seminal plasma.

In this study, using a gel overlay technique, we investigated calmodulin-binding proteins of epididymal and ejaculated bovine spermatozoa as well as seminal fluid. We have established that a previously well-characterized family of proteins of bull seminal plasma (BSP) designated as BSP-A1, BSP-A2, BSP-A3 and BSP-30 kDa (collectively called BSP proteins; Manjunath and Sairam, 1987; Manjunath *et al.*, 1987) and their tryptic fragments interact with calmodulin. Our previous

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studies have shown that these BSP proteins interact with high-density lipoprotein, phospholipids and heparin (Manjunath *et al.*, 1988, 1989; Chandonnet *et al.*, 1990; Desnoyers and Manjunath, 1992). In view of the binding properties exhibited by these BSP proteins, we have suggested that they play a role in sperm capacitation (Manjunath *et al.*, 1989; Chandonnet *et al.*, 1990; Desnoyers and Manjunath, 1992). The present study, which demonstrates that BSP proteins also interact with calmodulin, further substantiates this hypothesis. Preliminary results of these studies have recently been presented (Chandonnet *et al.*, 1989).

Materials and Methods

Materials

Phosphodiesterase 3',5'-cyclic nucleotide activator (calmodulin) from bovine brain was purchased from Sigma (St Louis, MO). Affinity-pure goat anti-rabbit IgG (H and L) was obtained from Jackson Immuno-research Laboratories Inc. (Avondale, PA). Na¹²⁵I was obtained from Amersham (Oakville, Ontario). Trypsin from bovine pancreas was purchased from Boehringer Mannheim (Germany). Acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS) and other electrophoresis products were obtained from Bio-Rad (Richmond, CA). Molecular mass markers of 14 to 94 kDa were purchased from Pharmacia (Canada). Nitrocellulose paper was purchased from Schleicher and Schull (Germany). All other chemicals used were of analytical grade and obtained from commercial suppliers unless otherwise indicated. BSP-A1, -A2, -A3 and -30 kDa proteins were isolated by gelatin-agarose affinity chromatography as described previously (Manjunath *et al.*, 1987). BSP-A1 and -A2 have an identical amino acid composition, but differ in their carbohydrate content. In this study, these proteins will be considered as a single chemical unit and will be referred to as BSP-A1/-A2.

Sperm preparation

Cauda epididymides with attached vasa deferentia were obtained from freshly killed bulls at a local slaughterhouse. The needle of a syringe was filled with light mineral oil and inserted into the lumen of the vas deferens and the light mineral oil was perfused retrograde into the epididymis. The epididymal tubule was then cut in one region and cells released were collected and washed three times (600 g, 20°C, 5 min) in phosphate-buffered saline (50 mmol sodium phosphate l⁻¹, pH 7.4 containing 0.9% NaCl). Spermatozoa were then routinely resuspended to give 40 × 10⁶ cells ml⁻¹, distributed in Eppendorf tubes in 1 ml fractions and pelleted. Proteins in the sperm pellet were then solubilized in the sample buffer as described by Laemmli (1970).

Semen from four bulls was donated by the Centre d'Insémination Artificielle du Québec (CIAQ Inc., St-Hyacinthe, Québec, Canada). The ejaculated spermatozoa were prepared as described above.

Iodination procedure

Affinity-pure goat anti-rabbit IgG and bovine brain calmodulin were iodinated with ¹²⁵I using the lactoperoxidase method as

described earlier (Manjunath and Sairam, 1982). Free iodine was removed by passing the reaction mixture through a Sephadex G-25 column equilibrated with phosphate-buffered saline containing 0.1% bovine serum albumin. The respective specific activities were usually between 60–80 µCi µg⁻¹ and 10 µCi µg⁻¹.

Calmodulin gel overlay procedure

Samples of proteins (5–30 µg) were separated electrophoretically on a 15% polyacrylamide gel (Laemmli, 1970). The calmodulin-binding proteins were detected using slight modifications of the procedure of Glenney and Weber (1983). After electrophoresis, the proteins in the gel matrix were fixed in a solvent mixture (methanol:acetic acid:water; 40:10:50) for 1 h and SDS in the gels was removed by overnight incubation in 10% ethanol. The gels were then saturated in the overlay solution (0.5% bovine serum albumin, 0.02 mol imidazole l⁻¹, 0.1 mol KCl l⁻¹ and 0.02% NaN₃, pH 7.0) for 3 h. The gels were then incubated for 16 h with 100 ml of overlay solution containing ¹²⁵I-labelled calmodulin (2 × 10⁶ c.p.m.). These incubations contained either 1 mmol CaCl₂ l⁻¹ or 1 mmol EGTA l⁻¹ and were performed at room temperature with gentle agitation. After incubation with ¹²⁵I-labelled calmodulin, the gels were washed for 1–2 days in five changes of overlay solution containing 1 mmol CaCl₂ l⁻¹ or 1 mmol EGTA l⁻¹. The gels were then dried and exposed for 10–70 h at –80°C to Fuji X-Ray film.

Electrophoresis and immunoblotting

BSP proteins or alcohol precipitates of bovine seminal plasma (10 µg) were separated electrophoretically on a 15% polyacrylamide gel according to Laemmli (1970). Separated proteins were transblotted onto nitrocellulose paper as described by Towbin *et al.* (1979). Nonspecific binding sites were blocked by incubating nitrocellulose papers in phosphate-buffered saline containing 0.05% Tween-20 and 0.1% bovine serum albumin. The sheets were then incubated with the respective purified antibodies to the BSP protein (Chandonnet *et al.*, 1990). The immunoreaction was visualized using an ¹²⁵I-labelled goat anti-rabbit IgG (H and L) (2 × 10⁶ c.p.m. per 50 cm²).

Proteolysis of BSP proteins

Samples of BSP proteins were dissolved in 0.05 mol ammonium bicarbonate l⁻¹ solution containing 0.1 mmol CaCl₂ l⁻¹. Trypsin from bovine pancreas was dissolved in the same solution and added to the BSP proteins (enzyme:substrate ratio of 1:100, w/w). Samples were then incubated at 37°C and aliquots were withdrawn at different times (10, 30, 60 and 120 min). Trypsin was inactivated by immediately boiling the samples for 10 min. The samples were then lyophilized and analysed for their calmodulin-binding properties by the ¹²⁵I-labelled calmodulin gel overlay technique as described above.

Fig. 1. Calmodulin-binding proteins in bovine spermatozoa and seminal plasma. Proteins extracted from 10×10^6 bull epididymal and ejaculated spermatozoa, and 30 μ g alcohol precipitates of seminal plasma were electrophoresed and subjected to the 125 I-labelled calmodulin overlay procedure. Autoradiograms of calmodulin-binding proteins in the presence of (a) 1 mmol $\text{Ca}^{2+} \text{ l}^{-1}$ and (b) 1 mmol EGTA l^{-1} . Lane 1, proteins extracted from epididymal spermatozoa; lane 2, proteins extracted from ejaculated spermatozoa; lane 3, alcohol precipitate of bovine seminal plasma. Molecular mass standards shown at the left are, from top to bottom, phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α -lactalbumin.

Results

Calmodulin-binding proteins in bovine spermatozoa and seminal plasma

The epididymal and ejaculated bull spermatozoa as well as the alcohol precipitates of bull seminal fluid contained many calmodulin-binding proteins as detected by the 125 I-labelled calmodulin gel overlay technique. Certain of these proteins exhibited higher binding in the absence of calcium while others exhibited greater binding in the presence of this divalent cation.

The major calmodulin-binding proteins of epididymal spermatozoa had the following molecular masses: > 200, 34, 32, 20 and 12 kDa (Fig. 1a, lane 1). The ejaculated spermatozoa contained all of these calmodulin-binding proteins and, in addition, they contained 57, 49 and 40 kDa calmodulin-binding proteins (Fig. 1a, lane 2). In contrast, seminal fluid contained two proteins (16 and 15 kDa) that bind high amounts of calmodulin and several other proteins (49 and 28 kDa) and many polypeptides with molecular masses between 6 and 17 kDa that exhibit low calmodulin-binding activity (Fig. 1, lane 3).

The epididymal spermatozoa contained 45, 34, 32, 28, 20, 18, 16 and 12–14 kDa proteins which bind calmodulin (Fig. 1b, lane 1). The major calmodulin-binding proteins of ejaculated spermatozoa had the following molecular masses: > 200, 49, 40, 34, 32, 30, 25, 20, 16 and 12–14 kDa (lane 2). Seminal fluid contained a 28 kDa protein with weak binding activity and

several polypeptides with molecular masses in the range of 6–17 kDa that bind calmodulin (lane 3). Several of these polypeptides exhibit low calmodulin-binding activity in the presence of Ca^{2+} , but higher binding in the absence of Ca^{2+} , notably those with molecular masses of 20, 18, 16 kDa and 12–14 kDa proteins in spermatozoa and the 6–17 kDa polypeptides present in seminal fluid.

Calmodulin-binding to BSP proteins

We have shown earlier that the BSP proteins present in bovine seminal fluid have molecular masses in the ranges of 15–17 kDa and 28–30 kDa. In view of the fact that several proteins from spermatozoa and seminal fluid with molecular masses < 30 kDa bind 125 I-labelled calmodulin (particularly in the absence of Ca^{2+}) it was suspected that this group also contained BSP proteins. The possibility that the purified BSP proteins also bind to calmodulin was therefore investigated. Proteins BSP-A1/-A2, -A3 and -30 kDa bind to calmodulin both in the presence and absence of Ca^{2+} (Fig. 2a, b), but the binding of calmodulin to these proteins was slightly higher in the absence of Ca^{2+} . The lower band in lane 3 (Fig. 2a, b) with a molecular mass of 14 kDa is a degradative product of the BSP-30 kDa protein that was generated during storage. This band was not stained with Coomassie Blue (Fig. 2c, lane 3), but showed high calmodulin-binding activity, particularly in the absence of Ca^{2+} . As indicated earlier, BSP-A1 and -A2 have the same primary sequence, but differ in their carbohydrate content. Usually they appear as closely migrating doublets (Fig. 2c, lane 1), but occasionally they migrate together as a single broad band as observed in the overlay experiment (Fig. 2a, b, lane 1).

Immunoblotting

Several polypeptides in the seminal fluid with molecular masses of 6–14 kDa bind to calmodulin (Fig. 1) and, therefore, we suspected that some of these polypeptides may be derived from BSP proteins. Immunoblots were performed to establish this. Alcohol precipitates of bovine seminal fluid and authentic BSP proteins were separated by SDS-PAGE, transblotted onto nitrocellulose membranes and subjected to immunodetection with the purified antibodies against BSP proteins (Fig. 3). The immunoblot experiments showed the presence of polypeptides in the seminal fluid (6–14 kDa) that crossreacted with anti-BSP-A1/-A2 (Fig. 3 (a), lane 1), anti-BSP-A3 (Fig. 3 (b), lane 1) and anti-BSP-30 kDa (Fig. 3 (c), lane 1). This experiment, therefore, indicated that some of the polypeptides of seminal fluid with molecular masses of 6–14 kDa are indeed derived from BSP proteins.

Binding of calmodulin to tryptic peptides of BSP proteins

Several polypeptides of bovine seminal fluid with molecular masses between 6 and 14 kDa crossreact with antibodies against BSP proteins (Fig. 3). This indicates that some of these polypeptides are derived from the BSP proteins, possibly by proteolytic activity present in the seminal fluid. We, therefore, investigated whether tryptic-peptides of BSP proteins also bind to calmodulin. Samples of purified BSP-A1/-A2, -A3 and

Fig. 2. Binding of ^{125}I -labelled calmodulin to BSP-A1/-A2, BSP-A3 and BSP-30 kDa proteins. Approximately 10 μg of purified bovine seminal plasma proteins were electrophoresed and subjected to the ^{125}I -labelled calmodulin overlay procedure. Autoradiograms of calmodulin-binding proteins in the presence of (a) 1 mmol $\text{CaCl}_2 \text{ l}^{-1}$ and (b) 1 mmol EDTA l^{-1} and (c) Coomassie blue-stained gel of purified BSP proteins. Lane 1, BSP-A1/-A2; lane 2, BSP-A3; lane 3, BSP-30 kDa.

Fig. 3. Immunoblotting of seminal plasma proteins and purified bovine seminal plasma (BSP) proteins. Polypeptides were separated by SDS-PAGE, transblotted to nitrocellulose and probed with (a) anti-BSP-A1/-A2, (b) anti-BSP-A3 and (c) anti-BSP-30 kDa. (a) Lane 1, alcohol precipitate of BSP; lane 2, authentic BSP-A1/-A2. (b) Lane 1, alcohol precipitate of bovine seminal plasma; lane 2, authentic BSP-A3. (c) Lane 1, alcohol precipitate of bovine seminal plasma; lane 2, authentic BSP-30 kDa.

-30 kDa proteins were incubated separately with trypsin for different periods and the resulting tryptic peptides were analysed by the ^{125}I -labelled calmodulin gel overlay procedure (Fig. 4). Tryptic peptides of BSP-A1/-A2 protein, which have molecular masses of 6–14 kDa, bound high amounts of calmodulin, in the presence or absence of Ca^{2+} (Fig. 4a, b, lanes 1 and 2). This binding was higher than that observed with native

BSP-A1/-A2 itself (Fig. 2a, b, lane 1). Similarly, tryptic peptides derived from BSP-30 kDa proteins also exhibited higher calmodulin-binding activity, particularly those polypeptides with molecular masses of 14 and 6–7 kDa (Fig. 4a, b, lanes 5 and 6). In contrast, the tryptic peptides of BSP-A3 exhibited no binding to calmodulin in the absence or presence of Ca^{2+} (Fig. 4a, b, lanes 3 and 4, respectively).

Fig. 4. Binding of ^{125}I -labelled calmodulin to tryptic peptides of bovine seminal plasma (BSP) proteins. Approximately 10 μg of tryptic peptides were electrophoresed and subjected to the ^{125}I -labelled calmodulin overlay procedure. Autoradiograms of calmodulin-binding tryptic peptides in the presence of (a) 1 mmol $\text{Ca}^{2+} \text{ l}^{-1}$ and (b) 1 mmol EGTA l^{-1} . Lanes 1 and 2 correspond to 10 and 120 min tryptic digests of BSP-A1/-A2, respectively; lanes 3 and 4 correspond to 10 and 120 min tryptic digests of BSP-A3, respectively; lanes 5 and 6 correspond to 10 and 120 min tryptic digests of BSP-30 kDa protein, respectively.

Discussion

Ejaculated mammalian spermatozoa must undergo a series of physiological and morphological changes in the female reproductive tract to acquire the ability to fertilize the ovum. The first of these processes, capacitation, is characterized by removal or modification of certain surface components as well as an increase in the permeability of the plasma membrane to metal ions (Bedford, 1969; Yanagimachi and Usui, 1974). This influx of ions triggers the acrosome reaction, which is characterized by the fusion of the plasma membrane and outer acrosomal membrane (Bedford, 1968). It has been postulated that an influx of extracellular calcium ion (Ca^{2+}) is a prerequisite for mammalian spermatozoa to undergo the acrosome reaction (reviewed by Langlais and Roberts, 1985; Yanagimachi, 1988). Calmodulin has been reported to stimulate various calcium-dependent enzymatic activities through specific associations with calmodulin-binding proteins (Cheung, 1980; Means *et al.*, 1982). It was therefore

suggested that calmodulin might serve as a link between calcium influx and the acrosome reaction. Thus, the identification and characterization of the calmodulin-binding proteins present in spermatozoa and in seminal fluid should aid in the understanding of the role of calmodulin in sperm function as well as the mechanism of its regulation.

The gel overlay procedure used in this study indicates the presence of several calmodulin-binding proteins in bovine epididymal and ejaculated spermatozoa. However, there were marked differences in the patterns, particularly in molecular masses and the amount of radiolabelled calmodulin bound to these proteins. The differences observed are probably because ejaculated spermatozoa are exposed to fluids from the accessory glands. It is possible that some of the secretory products that bind to the ejaculated spermatozoa may also interact with calmodulin. Alternatively, proteolytic enzymes in the seminal plasma may hydrolyse those calmodulin-binding proteins that are present on the sperm surface and thereby change their molecular mass as well as their affinity for calmodulin. Our results also indicate a difference in calmodulin binding in the presence and absence of Ca^{2+} ; certain proteins bind calmodulin preferentially in the presence of Ca^{2+} , whereas others bind calmodulin only in its absence. Increased binding of calmodulin in the absence of Ca^{2+} has also been observed with proteins in plasma membrane preparations (Noland *et al.*, 1985) and outer acrosomal membrane complexes of bovine epididymal spermatozoa (Olson *et al.*, 1985), and with proteins (21–14 kDa) of ejaculated boar spermatozoa (Camatini and Casale, 1987) and human spermatozoa (Aitken *et al.*, 1988). The reason for this increased binding of calmodulin in the absence of Ca^{2+} is not clear, but it has been suggested that it may have some significance in the compartmentalization of calmodulin depending upon the Ca^{2+} concentration (Noland *et al.*, 1985).

This study also revealed the presence of many calmodulin-binding proteins in bovine seminal plasma. A higher binding of calmodulin to these proteins was observed in the absence of Ca^{2+} , particularly to those polypeptides with molecular masses < 16 kDa. Since our previous studies have shown that a group of major proteins (BSP-A1, -A2 and -A3) of bovine seminal fluid have molecular masses in this range (Manjunath and Sairam, 1987), we investigated whether these proteins were calmodulin-binding proteins.

BSP-A1, -A2, -A3 and -30 kDa proteins are secretory products of the seminal vesicles (Manjunath *et al.*, 1987, 1988). The biochemical properties and structure of these proteins have been studied in detail (Manjunath, 1984; Kemme *et al.*, 1986; Manjunath and Sairam, 1987; Manjunath *et al.*, 1987, 1988; Seidah *et al.*, 1987). We found, in the present study, that all BSP proteins bind calmodulin in the presence and absence of Ca^{2+} . Interestingly, the proteolytic fragments of BSP-A1/-A2 and -30 kDa proteins exhibit higher calmodulin-binding activity than the native BSP proteins, particularly in the absence of Ca^{2+} . One explanation for this higher binding could be that, in the native state, the calmodulin-binding site may be buried within the protein, rendering it inaccessible to calmodulin and, upon proteolysis, this site becomes more accessible.

The binding of calmodulin to BSP proteins and their proteolytic fragments may have some significance in sperm function. We have reported earlier that BSP proteins bind to the sperm surface after ejaculation (Manjunath *et al.*, 1988).

Recently, we identified the binding sites on spermatozoa to be choline phospholipids (Desnoyers and Manjunath, 1992). In view of these studies, it is possible that part of the sperm-bound BSP protein may be inserted into the plasma membrane to establish the communication with calmodulin, which is bound to the inner surface of the plasma membrane and thus participates in the regulation of events involving Ca^{2+} transport during capacitation and the acrosome reaction. Heparin or glycosaminoglycans which bind to BSP proteins may also participate in such a function. Glycosaminoglycans in oviductal and follicular fluids induce sperm capacitation or the acrosome reaction in several animals including bulls (reviewed in Parrish, 1987; Miller and Ax, 1990), but the mechanism by which heparin induces capacitation is not known. Using a ^{125}I -labelled calmodulin gel overlay procedure, Leclerc *et al.* (1989, 1990) showed that ejaculated bovine spermatozoa contain specific calmodulin-binding proteins. Heparin induced a decrease in the binding of these proteins to calmodulin, which was positively correlated with the fertilization rate. In other studies, it has been reported that heparin induces Ca^{2+} uptake by sperm cells (Handrow *et al.*, 1986). In view of these results, Leclerc *et al.* (1989) suggested that, during sperm capacitation, Ca^{2+} uptake could follow the decreased binding of calmodulin to the specific sperm proteins. Since BSP proteins also bind to heparin (Chandonnet *et al.*, 1989), it is possible that this interaction could lead to decreased binding of calmodulin with the concomitant entry of Ca^{2+} into spermatozoa, thus initiating capacitation.

The present studies reveal that bovine spermatozoa and seminal fluid contain several calmodulin-binding proteins. Certain of these proteins bind calmodulin preferentially in the presence of Ca^{2+} , while others exhibit higher binding in the absence of Ca^{2+} . These studies also reveal that a family of closely related major proteins of bovine seminal plasma bind calmodulin. Limited proteolysis of these proteins enhanced this binding of calmodulin. The physiological significance of this increased binding remains to be determined. The interaction of BSP proteins with heparin shown in our previous studies (Chandonnet *et al.*, 1990) as well as their binding to calmodulin suggest that the BSP proteins are probably involved in sperm capacitation.

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