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Major Proteins of Bovine Seminal Plasma and High-Density Lipoprotein Induce Cholesterol Efflux from Epididymal Sperm[1]

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ABSTRACT

One of the hypotheses to explain the mechanism of capacitation involves the loss of sperm membrane cholesterol. Here, we studied whether or not the major proteins of bovine seminal plasma designated as BSP-A1, -A2, -A3, and -30-kDa (collectively called BSP proteins), which are implicated in sperm capacitation, induce cholesterol efflux. When epididymal sperm were labeled with [^{3}H]cholesterol and incubated with bovine seminal plasma (0.05–2%) or BSP proteins (20–120 μg/ml) for 8 h, the sperm lost [^{3}H]cholesterol (3.6-fold and 3-fold, respectively). The same results in the presence of BSP-A1/-A2 were obtained (3.5–fold) by direct determination of cholesterol on unlabeled epididymal sperm. Analysis of efflux particles by ultracentrifugation on a sucrose gradient revealed a single symmetrical peak of radioactivity at 1.14 g/ml. Immunoblotting of the fractions obtained from size-exclusion chromatography of the efflux particles showed that a portion of the BSP proteins were associated with [^{3}H]cholesterol. Heparin (12 μg/ml) alone did not stimulate cholesterol efflux. In contrast, high-density lipoprotein (HDL, 100 μg/ml) alone stimulated cholesterol efflux up to 3.1–fold after 8 h. When labeled epididymal sperm were preincubated for 20 min with BSP-A1/-A2 (120 μg/ml), washed, and incubated with HDL (100 μg/ml) for 8 h, the total cholesterol efflux of the sperm suspension was 51.8 ± 5.0% compared to 39.3 ± 1.2% when HDL alone was used. These results indicate that BSP proteins and HDL play an important role in the sperm sterol efflux that occurs during capacitation. Furthermore, the heparin-induced sperm capacitation did not involve the efflux of sperm membrane cholesterol.

INTRODUCTION

During their transit through the female genital tract, mammalian sperm must undergo the process of capacitation and the acrosome reaction (AR) before fertilization of the ovum [1–3]. Sperm capacitation is a multistep process that is not well understood and that involves several biochemical and ultrastructural changes in the sperm membrane (for review see [4]). Many studies have shown that sperm capacitation is accompanied by a change in the lipid composition of the sperm plasma membrane. More specifically, capacitation involves a decrease in the membrane cholesterol:phospholipid ratio [5]. These changes appear to be a reversible phenomenon that influences the fluidity and ionic permeability of the sperm membrane [6–8]. Cholesterol is the major free sterol in ejaculated bovine sperm [9]. Several studies have demonstrated that cholesterol influx reduces the rate of spontaneous AR [10, 11] and also inhibits fertilization (inhibits or delays capacitation) in many species including the rabbit [12], rat [13], mouse [14], guinea pig [11], human [15], and bovine [16, 17]. The influx of cholesterol also reverses capacitation of sperm: indeed, vesicles from rabbit seminal plasma containing cholesterol and liposomes containing cholesterol render capacitated sperm incapable of fertilizing eggs in vivo [18]. On the other hand, Ehrenwald et al. [16, 17] have shown that the efflux of membrane cholesterol leads to bovine sperm capacitation and predisposes the sperm to penetrate ova at a higher rate than those sperm without reduced cholesterol levels. Recently, Zarintash and Cross [19] have shown that as human sperm lose their unesterified cholesterol they become responsive to progesterone (progesterone induces the AR in human sperm).

The net cholesterol efflux that is observed during capacitation is affected by the capacity of the medium to bind cholesterol. Several lines of evidence indicate that there are components in the female genital tract that facilitate this efflux of cholesterol. Many studies have shown that albumin [13, 14, 20, 21] and high-density lipoprotein (HDL) [22, 23] are acceptors of cholesterol. HDL, the only class of lipoprotein present in bovine and human oviductal and follicular fluid, appears to be a more efficient acceptor of cholesterol than albumin [22–25]. Therefore, HDL appears to play an important role in the sperm sterol modification that occurs during an early step of capacitation.

Bovine seminal plasma contains a family of closely related major proteins designated as BSP-A1, BSP-A2, BSP-A3, and BSP-30-kDa (collectively called BSP proteins). These BSP proteins are secretory products of seminal vesicles; they bind to sperm upon ejaculation and play a role in capacitation [26–30]. BSP-A1, -A2, and -A3 have molecular masses of 15–16.5 kDa, whereas BSP-30-kDa has a molecular mass of 28–30 kDa [29]. BSP-A1 and BSP-A2 are considered to be a single chemical entity named BSP-A1/-A2 (also called gonadostatins or PDC-109) [31] since they have an identical amino acid sequence but differ only in their degree of glycosylation. Upon ejaculation, the BSP proteins interact with the choline phospholipids on the sperm membrane [32]. These BSP proteins also bind to heparin, a glycosaminoglycan also implicated in capacitation [33], and to apolipoprotein A-I in free form or associated with HDL [29, 34, 35]. Recently, we have shown that the BSP proteins accelerate the capacitation of bovine epididymal sperm induced by heparin and HDL [36, 37]. In order to gain further insight into the mechanisms of sperm capacitation mediated by BSP proteins and capacitating factors (heparin and HDL), we investigated whether or not these factors stimulate cholesterol efflux from the plasma membrane of sperm during capacitation. The results showed that the BSP proteins and HDL play an important role in the efflux of cholesterol that occurs during capacitation.

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MATERIALS AND METHODS

Materials

BSA (fraction V fatty acid-free), taurine, L-epinephrine, erythrosin B, flavianic acid (naphthol), heparin (purified from porcine intestinal mucosa), and lysophosphatidylcholine (lyso-PC; purified from egg yolk) were from Sigma (St. Louis, MO); penicillin G and streptomycin sulfate were from Gibco (Burlington, ON, Canada); [^{3}H]cholesterol was from DuPont Canada (Mississauga, ON, Canada); transferrin was from Boehringer Mannheim (Laval, PQ, Canada); and Sepharose CL-4B was purchased from Pharmacia Biotech (Uppsala, Sweden). All other chemicals used were of analytical grade and obtained from commercial suppliers.

Bovine testes and epididymides were obtained from Abattoir Les Cèdres (St-Lazare, PQ, Canada), and freshly ejaculated bovine semen collected with an artificial vagina was obtained from the Centre d'Insémination Artificielle du Québec (St-Hyacinthe, PQ, Canada). The semen was centrifuged at 5000 $\times$ *g* for 10 min, and the seminal plasma was aspirated and then re-centrifuged at 10 000 $\times$ *g* for 15 min before the supernatant was used or for further purification of BSP proteins. BSP-A1/-A2, -A3, and -30-kDa were isolated using gelatin-agarose affinity chromatography [28]. The adsorbed fractions were eluted and resolved on Sephadex G-75 and G-200 columns under conditions described previously [27]. The purity of the BSP proteins was confirmed by SDS-PAGE.

Separation of HDL

HDL was isolated from human serum by density gradient ultracentrifugation [37]. The purity of the HDL was assessed by the Lipo-Gel kit following the manufacturer's instructions (Beckman Instruments, Fullerton, CA).

Sperm Labeling and Cholesterol Efflux

The medium used for washing and incubation of sperm was a modified Tyrode's albumin lactate pyruvate (TALP) medium, designated mTALP [36]. Caudal sperm were collected by retroperfusion of the epididymides obtained from four different bulls, pooled, and washed 3 times (350 $\times$ *g*, 10 min) with 10 vol of mTALP. The sperm were then labeled using the method described by Langlais et al. [23] with minor modifications. At the end of the incubation (conducted at 39°C instead of 37°C), sperm labeled with [^{3}H]cholesterol were washed 3 times (350 $\times$ *g*, 10 min) and resuspended in 5 ml of medium. The sperm were incubated for 1 h at 39°C under 5% CO_2 for equilibration of the label, centrifuged, and resuspended in medium to obtain 1×10^8 cells/ml. Approximately 50–60% of [^{3}H]cholesterol was incorporated into sperm and was $< 0.11\%$ of the sperm cholesterol mass. This value is comparable to those reported (60–65%) previously [23]. Studies of sperm cholesterol efflux were done by incubating 500 μl of the labeled sperm suspension with 500 μl of medium alone or 500 μl of medium containing different concentrations of bovine seminal plasma or of BSP proteins. The sperm were then incubated for 8 h at 39°C under 5% CO_2, during which two samples of 50 μl were withdrawn at different times to determine the percentage of [^{3}H]cholesterol taken up by the incubation medium. The percentage of [^{3}H]cholesterol released into the medium was calculated from the radioactivity retrieved in the incubation medium divided by the total radioactivity associated with the sperm in the suspension. In another set of experiments, labeled sperm were incubated in the presence or absence of BSP-A1/-A2 for 20 min, washed twice (350 $\times$ *g*, 10 min), and incubated for 8 h at 39°C under 5% CO_2 in the presence or absence of 100 μg/ml of HDL or 12 μg/ml of heparin. Experiments were done under similar conditions in the presence of BSA and transferrin, which served as control proteins.

Density Gradient Ultracentrifugation

After cholesterol efflux in the presence of 40 μg/ml of BSP proteins (8-h incubation), the incubation medium containing the efflux particles was centrifuged twice (940 $\times$ *g*, 15 min) to remove sperm. The supernatant (1 ml) was applied to a sucrose gradient (1.5 ml of 65% sucrose and 2 ml of 34% sucrose in PBS pH 7.4). The samples were then centrifuged at 50 000 rpm for 24 h at 18°C (rotor AH-650; Sorval Instrument, DuPont, Wilmington, DE). After ultracentrifugation, the samples were fractionated into 225-μl aliquots. The amount of radioactivity present in each fraction was then determined using a beta-counter, and the density was determined by the refractive index measured with a refractometer (ABBE-3L; Milton Roy, Rochester, NY).

Chromatographic Analysis

After cholesterol efflux in the presence of 120 μg/ml of BSP-A1/-A2 (8-h incubation), the incubation medium containing the efflux particles was centrifuged twice (940 $\times$ *g*, 15 min) to remove sperm. The supernatant (1 ml) was applied to a Sepharose CL-4B column (2.5 $\times$ 90 cm) previously washed with 50 mM PBS buffer (pH 7.4). The elution was carried out at a flow rate of 50 ml/h with the same buffer. Fractions of 2.5 ml were collected, and the radioactivity was determined using a beta-counter. One hundred microliters of each fraction was treated with trichloroacetic acid (TCA, final concentration of 15%) to precipitate the proteins, which were then analyzed by SDS-PAGE and immunoblotting.

SDS-PAGE Analysis and Immunoblotting

SDS-PAGE in 15% polyacrylamide gels was performed as described previously [38]. The gels were then transferred to nitrocellulose membranes as described by Towbin et al. [39], and immunodetection using affinity-purified polyclonal antibodies against BSP-A1/-A2 was done as previously described [40] by using an enhanced chemiluminescence reagent kit (Mandel Scientific, Guelph, ON, Canada) for detection.

Determination of Sperm Cholesterol

Washed unlabeled epididymal sperm (final concentration of 5×10^7 cells/ml) were incubated for 8 h in the presence or absence of 120 μg/ml of BSP-A1/-A2. After incubation, the sperm suspension was centrifuged (940 $\times$ *g*, 15 min) to remove the supernatant, which contained the efflux particles. Then the sperm pellet was incubated for 20 min with 200 mM of choline in mTALP to release BSP-A1/-A2 bound to sperm in order to prevent interference with the lipid extraction. After incubation, sperm lipids were extracted using chloroform/methanol (2:1) [41]. After evaporation of solvent with N_2, the lipids were resuspended in isopropanol, and the amount of cholesterol was determined using the cholesterol determination kit (Boehringer Mannheim; cat. no. 139 050) and following the protocol described by the manufacturer.

Sperm Capacitation and AR

To determine the number of capacitated sperm after the efflux studies, lyso-PC was added at 100 μg/ml, and the sperm were reincubated for an additional 15 min. This concentration of lyso-PC was previously shown to induce the AR in capacitated sperm while having no effect on non-capacitated sperm [33]. The lyso-PC-induced AR is a well-characterized method that has been correlated with in vitro fertilization rates and validated by electron micrograph studies [33]. Before drying and staining, randomly selected slides were examined using light microscopy to verify sperm motility. The percentage of sperm that were acrosome-reacted was determined on air-dried sperm smears with a naphthol yellow-erythrosin B-staining procedure [42], and viability was estimated by the viability staining protocol of Dott and Foster [43].

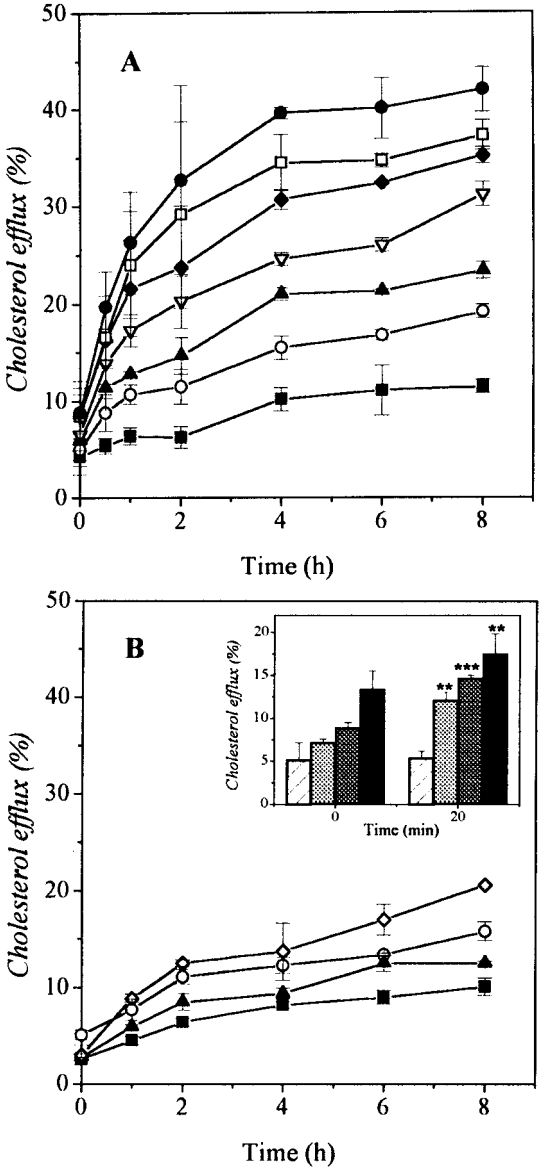

FIG. 1. Cholesterol efflux from bovine epididymal sperm in the presence of seminal plasma. **A**) Labeled ([^{3}H]cholesterol) epididymal sperm were incubated for 8 h with bovine seminal plasma or culture medium: 0% (control; solid squares); 0.05% (open circles); 0.1% (solid triangles); 0.25% (inverted triangles); 0.5% (diamonds); 1% (open squares), and 2% (solid circles) seminal plasma. The cholesterol efflux in the presence of more than 0.1% of bovine seminal plasma was significantly different from control ($p < 0.05$). **B**) Labeled ([^{3}H]cholesterol) epididymal sperm were incubated for 20 min with bovine seminal plasma (inset: 0%, white [control, culture medium only]; 0.5%, light gray; 2%, gray; 25%, black), washed twice (350 × *g*, 10 min), and incubated with culture medium for 8 h. 0% (control, squares); 0.5% (circles); 2% (triangles), and 25% (diamonds). The percentage of cholesterol efflux at each time point was calculated as described in *Materials and Methods*. Results represent the mean ± SEM of three independent experiments performed in triplicate. Significant differences vs. control (without seminal plasma, inset): $^{**}p < 0.01$; $^{***}p < 0.005$. The cholesterol efflux in the presence of more than 25% of bovine seminal plasma was significantly different from control (without seminal plasma; $p < 0.05$).

Protein Assay

The protein content of the samples was measured according to the method of Bradford [44] or by weighing freeze-dried purified proteins on a Cahn microbalance (Model C-31; Fisher Scientific, Fairlawn, NJ).

Data Analysis

The data presented here were analyzed for significant differences by covariance analysis or by a Student's *t*-test on paired observations.

RESULTS

Cholesterol Efflux from Bovine Epididymal Sperm in the Presence of Seminal Plasma

To study the sperm cholesterol efflux, spermatozoa were labeled with [H^3]cholesterol, and increasing concentrations of whole bovine seminal plasma were added to the medium. The appearance of cholesterol in the medium was determined at different times (see *Materials and Methods*). As shown in Figure 1A, the whole seminal plasma stimulated sperm cholesterol efflux in a dose-dependent manner. The rate of sperm cholesterol efflux was rapid in the first 2 h and then reached a plateau after 4 h. Whole seminal plasma (2%, 8 h) increased sperm [^{3}H]cholesterol efflux to 42.0 ± 2.3% compared to control without seminal plasma (11.5 ± 0.7%). Since seminal plasma gradually becomes diluted in the female genital tract fluid and those sperm that are in transit through the female genital tract gradually escape from seminal fluid, in the next experiment labeled sperm were preincubated 20 min with whole seminal plasma (0.5%, 2%, and 25%), washed twice, and then reincubated for 8 h in culture medium (Fig. 1B). During the first 20 min of incubation with whole seminal plasma, the efflux of [^{3}H]cholesterol was rapid (Fig. 1B, inset), as observed in the previous experiment (Fig. 1A). When the spermatozoa were washed twice to remove those proteins that were not bound to the sperm membrane and then incubated with protein-free medium, the capacity of whole seminal plasma to stimulate cholesterol efflux decreased (only 20.6% after 8 h using 25% seminal plasma, Fig. 1B). For all of the experiments, the epididymal sperm viability and motility of treated sperm were the same as those observed in the control sample (medium alone; data not shown).

Cholesterol Efflux from Bovine Epididymal Sperm in the Presence of BSP Proteins

The BSP proteins constitute 60–70% of the proteins in bovine seminal plasma; therefore in the next experiment we used purified BSP proteins to verify whether these proteins were responsible for the sperm cholesterol efflux stimulation effect of seminal plasma. When we used increasing

TABLE 1. AR of labeled epididymal sperm incubated 6 h in the presence or absence of purified BSP proteins (120 μg/ml).

Treatments[a]	AR (%)[b,c]
Medium alone	15.1 ± 0.4
BSP-A1/-A2	33.8 ± 1.4
BSP-A3	38.5 ± 0.3
BSP-30-kDa	33.5 ± 0.5

[a] Labeled epididymal sperm were incubated for 6 h in the presence or absence of purified BSP proteins before lyso-PC was added to induce the AR.
[b] Results represent the mean ± SEM of two independent experiments that included three samples per experiment and in which 200 sperm were assayed per sample.
[c] Increases of AR stimulation by purified BSP proteins are significant vs. control sample (without BSP proteins, $p < 0.001$).

concentrations of BSP proteins, we obtained approximately the same stimulation of cholesterol efflux (Fig. 2) as found with bovine seminal plasma. The efflux of sperm [^{3}H]cholesterol was rapid during the first 2 h and then reached a plateau. BSP-A1/-A2 stimulated the efflux of sperm cholesterol in a dose-dependent manner, with a maximum stimulation of 32.4 ± 1.3% when 120 μg/ml of protein were used. This concentration of proteins corresponds to the quantity of BSP proteins present in 0.25–0.5% of bovine seminal plasma. Similar results were obtained with BSP-30-kDa and BSP-A3 (Fig. 2B). The efflux curve obtained in the presence of BSP-A3 was slightly different from that obtained with BSP-A1/-A2 and BSP-30-kDa. However, after 8 h, each purified BSP protein provided a similar percentage of cholesterol efflux. After 6 h of incubation, an aliquot of labeled sperm suspension of the control sample as well as that incubated with purified BSP proteins was taken to evaluate sperm capacitation (Table 1). After a 6-h incubation, all the purified BSP proteins stimulated the AR of labeled sperm (33.5% to 38.5%) compared to control (15.1 ± 0.4%). Lyso-PC, which induces the AR only in capacitated sperm, was used as an indirect method to measure sperm capacitation (see *Materials and Methods*). Therefore, the AR in the present context is meant to reflect capacitation rather than the AR.

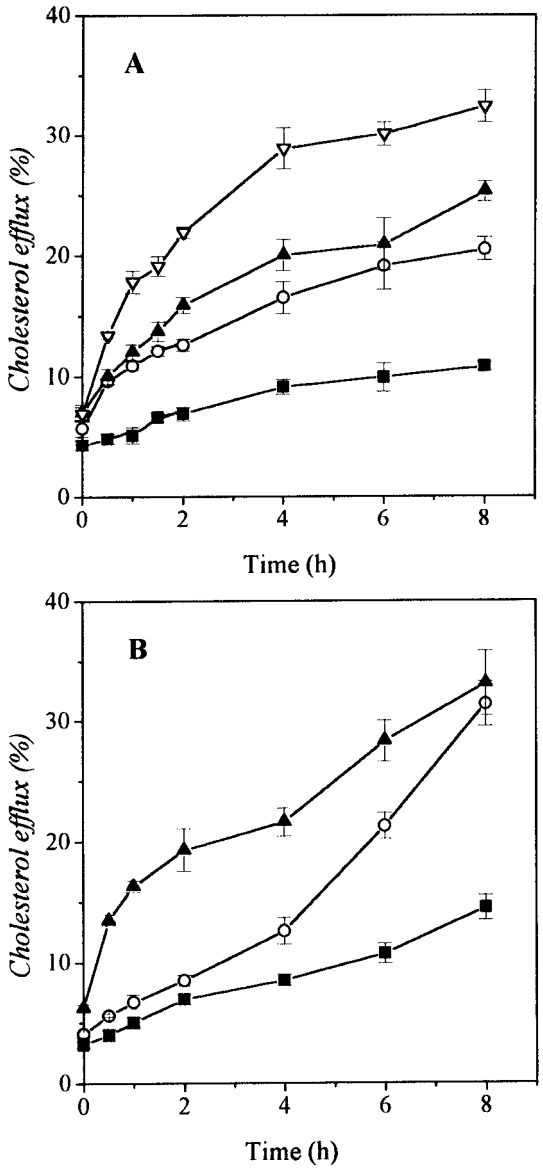

FIG. 2. Cholesterol efflux from bovine epididymal sperm in the presence of BSP proteins. The same protocol was used as described in Figure 1A using purified BSP proteins instead of seminal plasma. **A**) BSP-A1/-A2 (20 μg/ml, circles, 40 μg/ml, solid triangles and 120 μg/ml, inverted triangles) or culture medium (control, squares). **B**) BSP-A3 (120 μg/ml, circles) and BSP-30-kDa (120 μg/ml, triangles) or culture medium (control, squares). Results represent the mean ± SEM of three (**A**) or two (**B**) independent experiments performed in triplicate. The cholesterol efflux in the presence of more than 20 μg/ml of BSP-A1/-A2 or in the presence of BSP-A3 (120 μg/ml) or BSP-30 kDa (120 μg/ml) was significantly different from control (without BSP proteins, $p < 0.025$).

Density Gradient Ultracentrifugation Analysis

To analyze the lipid efflux particles generated in the presence of BSP proteins, labeled sperm were incubated for 8 h in the presence or absence of purified BSP proteins. The incubation medium containing the efflux particles was subjected to sucrose gradient ultracentrifugation. In the presence of purified BSP proteins, we observed a single symmetrical peak of radioactivity at 1.14 g/ml (Fig. 3, B–D).

Chromatographic Analysis of the Efflux Particle

To verify whether the efflux particles contained BSP proteins associated with [^{3}H]cholesterol, chromatographic analysis, SDS-PAGE analysis, and immunoblotting were used. After cholesterol efflux in the presence of BSP-A1/-A2 (more abundant BSP proteins), the incubation medium containing the efflux particles was chromatographed on a Sepharose CL-4B column (Fig. 4A). A single peak of radioactivity was obtained (fractions 12–20), and a single detectable peak of protein that corresponded to the BSA present in the medium was also obtained (fractions 59–67). The proteins of each fraction were analyzed by immunoblotting using affinity-purified antibody against BSP-A1/-A2 (Fig. 4B). The results showed that a portion of BSP-A1/-A2 was detected in fraction 14, and the major part was detected in fractions 54–75.

Cholesterol Efflux from Unlabeled Epididymal Sperm With or Without BSP Proteins

To confirm whether BSP proteins specifically remove sperm cholesterol, washed epididymal sperm that were not labeled with cholesterol were incubated for 8 h with or without BSP-A1/-A2. Before the incubation, a fraction was taken to determine the amount of cholesterol present in epididymal

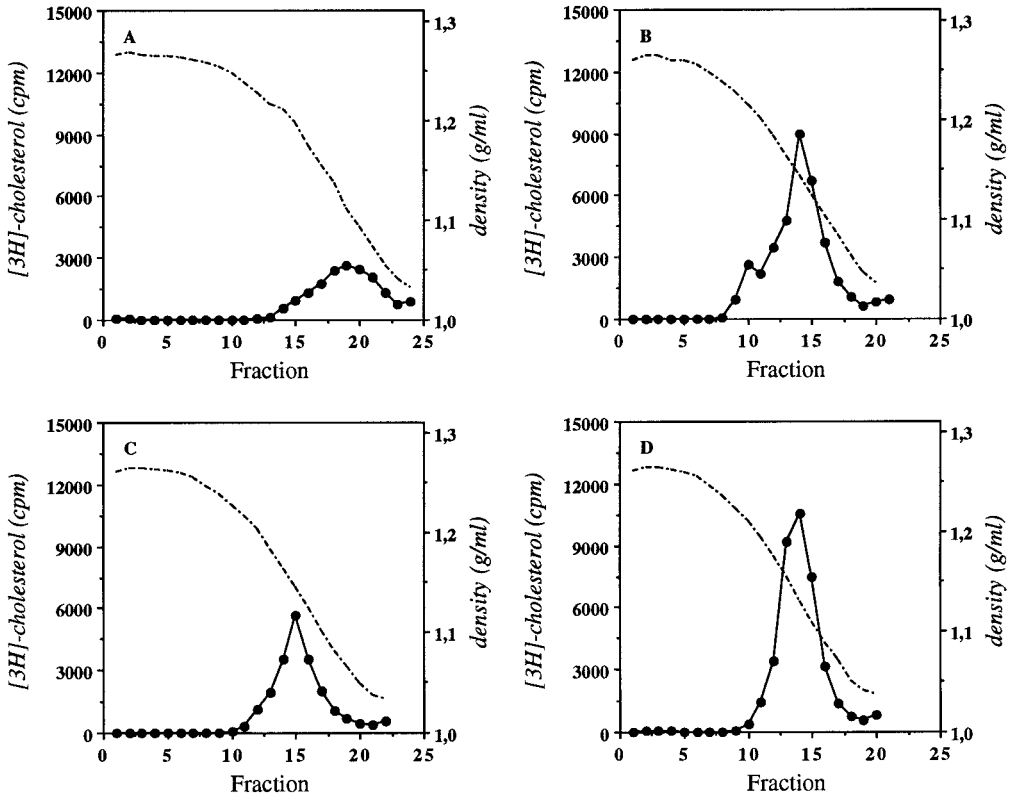

FIG. 3. Analysis by density gradient ultracentrifugation of lipid particles recovered in the incubation medium after [^{3}H]cholesterol efflux in the presence of BSP proteins. Labeled ([^{3}H]cholesterol) epididymal sperm were incubated for 8 h with 40 μg/ml of purified BSP proteins BSP-A1/-A2 (**B**), BSP-A3 (**C**), and BSP-30-kDa (**D**), or with culture medium (control, **A**). After incubation, the samples were centrifuged twice (940 × *g*, 15 min). An aliquot (1 ml) of each supernatant was analyzed by sucrose density gradient ultracentrifugation. The samples were fractionated (225 μl), and the radioactivity in each fraction was determined. The density was determined on the basis of the refractive index.

sperm (246.1 ± 16.0 μg cholesterol/1 × 10^9 sperm). After incubation with or without BSP-A1/-A2, spermatozoa were centrifuged and incubated with choline to release any BSP-A1/-A2 bound to sperm (to prevent interference with subsequent lipid extraction), and sperm cholesterol was extracted and quantified. After 8 h of incubation, spermatozoa that were incubated with BSP-A1/-A2 lost 19.1% of their cholesterol compared to spermatozoa that were incubated with medium alone, which lost only 5.4% (Table 2).

Cholesterol Efflux from Bovine Epididymal Sperm in the Presence of BSP-A1/-A2 and Capacitating Agents

Several studies have shown that the HDL present in the female reproductive tract can facilitate the sperm cholesterol efflux that occurs during capacitation [22, 23]. Heparin is another important factor that is also implicated in bovine sperm capacitation [33, 36]. In the next experiment, we investigated whether or not these capacitating factors could stimulate sperm cholesterol efflux in the presence or absence of BSP-A1/-A2. For these experiments, labeled sperm were preincubated with BSP-A1/-A2 or medium alone for 20 min. This period of time is sufficient for maximum binding of BSP-A1/-A2 to the sperm membrane [45, 46]. During the first 20 min, BSP-A1/-A2 stimulated cholesterol efflux (17.3 ± 4.0% compared to the control 6.9 ± 0.6%, Fig. 5A, inset). After this preincubation, sperm were washed twice and then incubated with heparin or HDL for 8 h. After washing, which removed the proteins that were not bound to sperm, bound BSP-A1/-A2 stimulated the cholesterol efflux only weakly (Fig. 5, A and B). Likewise, heparin alone or preincubated in the presence of BSP-A1/-A2 did not stimulate sperm cholesterol efflux (Fig. 5A). On the other hand, HDL alone continuously stimulated the cholesterol efflux; no plateau was reached even after 8 h of incubation (32.4 ± 0.6% cholesterol efflux, Fig. 5B). The efficiency of HDL in promoting cholesterol efflux was similar from sperm preincubated with or without BSP-A1/-A2. However, the total cholesterol efflux of the sperm suspension (20 min + 8 h) was more substantial in the presence of BSP-A1/-A2 and HDL (51.8 ± 5.0%) than of HDL alone (39.3 ± 1.2%).

The medium, mTALP, used in each treatment contained 0.6% BSA (6 mg/ml); this concentration of BSA barely stimulated sperm cholesterol efflux (Figs. 1, 2, and 5, control medium alone). When higher concentrations of BSA

TABLE 2. Cholesterol content of epididymal sperm before and after 8-h incubation with BSP-A1/-A2.

Treatments[a]	Cholesterol/ 1×10^9 sperm[b,c] (μg)	Cholesterol loss (%)
Epididymal sperm (no treatment)	246.1 ± 16.0	–
Epididymal sperm incubated with medium alone	232.8 ± 14.4	5.4
Epididymal sperm incubated with BSP-A1/-A2 (120 μg/ml)	199.2 ± 7.5	19.1

[a] Unlabeled epididymal sperm were incubated for 8 h in the presence or absence of BSP-A1/A2 before determination of sperm cholesterol.
[b] Results represent the mean ± SEM of two independent experiments that included two samples per experiment.
[c] Decreases of sperm cholesterol content obtained in the presence of BSP-A1/-A2 are significant vs. epididymal sperm not incubated ($p < 0.05$).

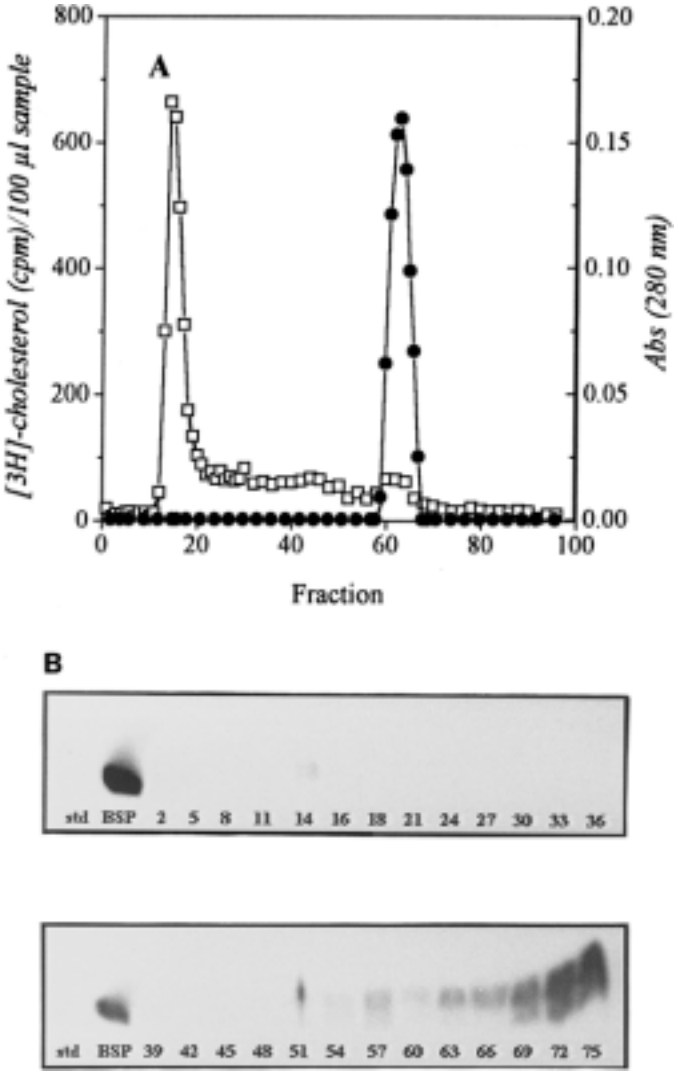

FIG. 4. Chromatographic analysis of the efflux particles. Labeled ([^{3}H]cholesterol) epididymal sperm were incubated for 8 h with 120 µg/ml of purified BSP-A1/-A2. Then the incubation medium containing the efflux particles was centrifuged to remove spermatozoa. The supernatant (1 ml) was applied to a Sepharose CL-4B column. After 60 min, fractions of 2.5 ml were collected, and the absorbance (280 nm, circles) and the radioactivity (using a beta-counter, squares) were determined (**A**). One hundred microliters of certain fractions were treated with 15% TCA to precipitate the proteins, which were then analyzed by SDS-PAGE and immunoblotting (**B**).

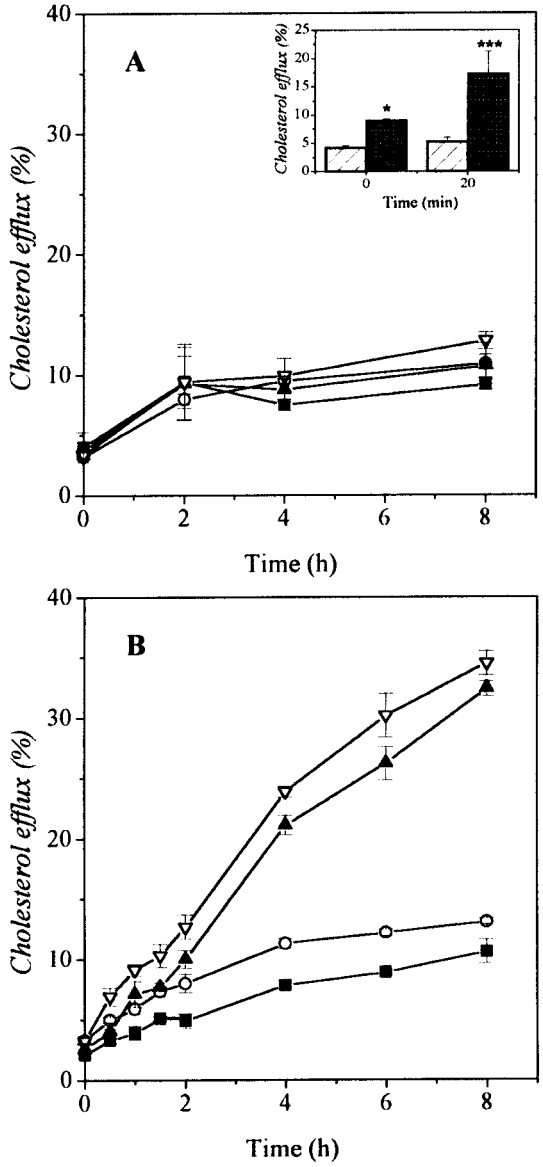

FIG. 5. Cholesterol efflux from bovine epididymal sperm in the presence of BSP-A1/-A2 and capacitating agents. Labeled ([^{3}H]cholesterol) epididymal sperm were incubated for 20 min with 120 µg/ml of BSP-A1/-A2 (inset: black) or culture medium (inset: control, white), washed twice (350 × *g*, 10 min), and incubated for 8 h with or without heparin (**A**) or HDL (**B**). **A**) Medium alone (control, squares); BSP-A1/-A2 alone (120 µg/ml, circles); heparin alone (12 µg/ml, solid triangles), and BSP-A1/-A2 (120 µg/ml) and heparin (12 µg/ml, inverted triangles). Significant differences vs. control (without BSP-A1/-A2, inset): $*p < 0.05$; $***p < 0.005$. No significant difference vs. control (medium alone) were observed. **B**) Medium alone (control, squares); BSP-A1/-A2 alone (120 µg/ml, circles); HDL alone (100 µg/ml, solid triangles), and BSP-A1/-A2 (120 µg/ml) and HDL (100 µg/ml, inverted triangles). Results represent the mean ± SEM of two (**A**) or three (**B**) independent experiments performed in triplicate. The cholesterol efflux in the presence of HDL alone or in the presence of HDL and BSP-A1/-A2 was significantly different vs. control (medium alone; $p < 0.005$).

were used (20 and 50 mg/ml), the efflux of cholesterol did not exceed 16.2 ± 1.1% after 8 h (data not shown). As a protein control, transferrin was also tested for the stimulation of cholesterol efflux. The maximal cholesterol efflux value obtained in the presence of transferrin (data not shown) was not significantly different from control (medium alone).

DISCUSSION

In mammals, loss of sperm cholesterol during capacitation has been demonstrated to occur in many species including cattle, rabbit, mouse, and human [14, 16, 22, 23]. The magnitude of net cholesterol efflux is affected by the cholesterol-binding capacity of those components present in the follicular and oviductal fluids (HDL and BSA) [13, 14, 21–23]. The present results confirm that HDL facilitates the efflux of sperm cholesterol, but we also demonstrated for the first time that the BSP proteins stimulate cholesterol efflux from the bovine sperm membranes. At the higher concentration of whole bovine seminal plasma used (2%, 8 h), 42.0 ± 2.3% of sperm [^{3}H]cholesterol was removed from the sperm compared to control (11.5 ± 0.7%; Fig. 1A). Our results also indicate that the BSP proteins are responsible for this efflux stimulation (Fig. 2 and Table 2). The cholesterol efflux mediated by BSP proteins (120 µg/

ml) and 0.25–0.5% of whole seminal plasma (which contains approximately the same amount of BSP proteins) after 8 h were similar (31–35%, Figs. 1A and 2). The efflux curves obtained in the presence of whole seminal plasma and BSP proteins were also similar; the rate of cholesterol efflux was rapid in the first 2 h and then reached a plateau after 4 h. Comparable results were obtained when we preincubated the sperm for 20 min with whole seminal plasma or with BSP proteins, washed twice and then incubated with medium alone. In these experiments, the whole seminal plasma and the BSP proteins stimulated the cholesterol efflux only weakly when sperm were washed, i.e., when whole seminal plasma or BSP proteins in the media were removed (Fig. 1B and Fig. 5). All of these results confirm that the BSP proteins are the major elements of bovine seminal plasma responsible for the stimulation of sperm cholesterol efflux.

The efflux particles obtained in the presence of BSP proteins were homogeneous particles with a density comparable to that of HDL [22] (Fig. 3). These particles contained BSP proteins associated with [^{3}H]cholesterol (Fig. 4). Therefore, the BSP proteins can form a complex with cholesterol, but only a small portion of BSP proteins participated in the formation of the efflux particles. A previous study has shown that BSP proteins cannot bind to immobilized cholesterol [32]. However, we [27] and others [47] have reported that the BSP proteins form aggregates (60 000–150 000 daltons). These aggregates may exhibit hydrophobic cavities in which cholesterol could be trapped. Further studies are required to characterize the nature of the efflux particles and to determine the proportion of BSP proteins and cholesterol present in the efflux particles.

To evaluate unambiguously that BSP proteins stimulate cholesterol efflux, we used two different methods: labeled sperm with [^{3}H]cholesterol (Fig. 2) and direct determination of the amount of sperm cholesterol before and after incubation with BSP-A1/-A2 (Table 2). Labeled sperm incubated for 8 h in the presence of 120 μg/ml of BSP-A1/-A2 stimulated up to 32.4 ± 1.3% of cholesterol efflux compared to the control sample (medium alone, 10.8 ± 0.4%). By direct determination of sperm cholesterol, those sperm that were incubated with BSP-A1/-A2 lost 19.1% of their cholesterol compared to control (5.4%). The percentage of cholesterol efflux obtained with labeled sperm was higher than that of direct determination. However, when we compared the amount of cholesterol efflux induced by BSP-A1/-A2 to that found in the control sample, the two methods provided similar results: a 3-fold stimulation in the presence of BSP-A1/-A2 compared to control when labeled sperm were used, and a stimulation of 3.5-fold compared to the control when direct determination of sperm cholesterol was used.

Our previous results [37] have shown that bovine epididymal sperm incubated for 20 min with BSP-A1/-A2, washed twice, and then incubated for 8 h with medium alone did not capacitate sperm. In the present study, when we performed similar experiments and determined cholesterol efflux, we observed that during the first 20 min, BSP-A1/-A2 stimulated cholesterol efflux (17.3 ± 4.0%, Fig. 5A, inset). This result indicates that the cholesterol efflux that occurred during the 20 min of incubation with BSP-A1/-A2 was probably not sufficient to stimulate sperm capacitation, but continuous exposure of sperm to BSP proteins led to 30–33% of cholesterol efflux, and this resulted in a 2-fold higher capacitation than that observed in the control sample (Table 1). Therefore, BSP proteins alone would appear to be sufficient to capacitate bovine epididymal sperm when incubated with them for longer periods, but this situation is not physiological, since seminal plasma that contains BSP proteins is gradually diluted and lost during sperm transit through the female genital tract [48].

The environment of the female genital tract provides conditions that also promote efflux of cholesterol from the sperm membrane. In the bovine species, HDL, probably derived from plasma, is the only class of lipoprotein present in follicular and oviductal fluids [22, 24, 25]. Oviductal HDL is elevated during the estrous period and remains low throughout the rest of the estrous cycle [7]. Compared to plasma HDL, follicular fluid HDL contains a higher proportion of pre-β-HDL [25], which has been demonstrated to play a major role as first cholesterol acceptor [49, 50]. Our previous studies have shown that BSP proteins stimulate epididymal sperm capacitation induced by heparin and HDL [36, 37]. In these studies, epididymal sperm incubated for 20 min with BSP proteins, washed twice, and incubated for 5 h with heparin capacitated 2- to 3-fold more spermatozoa compared to control without incubation with BSP proteins [36]. When HDL was used instead of heparin, HDL alone stimulated capacitation of epididymal sperm after 8 h, but the preincubation with BSP proteins (20 min) increased the percentage of sperm capacitation and decreased the time required to 5 h for a significant increase in capacitation [37]. Here, we report that HDL alone continuously stimulated sperm cholesterol efflux (Fig. 5B), and this efflux was not affected by the presence of BSP-A1/-A2. Therefore, HDL and BSP-A1/-A2 probably stimulate cholesterol efflux independently. However, the total sperm cholesterol efflux was higher in the presence of BSP-A1/-A2 (20 min) and HDL (8 h, 51.8 ± 5.0%) than when HDL alone (20 min with medium alone, then 8 h with HDL) was used (39.3 ± 1.2%). Thus, these results suggest that the mechanism by which HDL and BSP proteins stimulate capacitation involves cholesterol efflux. BSA represents the second cholesterol acceptor present in the bovine female genital tract, and it constitutes more than 90% of the total oviductal fluid protein [22]. The concentration of albumin in oviductal fluid changes during the estrous cycle and varies between 6.2 and 19.2 mg/ml [22]. Our results with BSA confirm the result obtained previously by Ehrenwald et al. [22] that HDL is the most efficient acceptor of cholesterol in oviductal fluid.

When heparin was used instead of HDL, heparin in the presence or absence of BSP-A1/-A2 did not stimulate sperm cholesterol efflux (Fig. 5A). These results confirm that the capacitation induced by heparin in the presence of BSP proteins proceeds by a different mechanism than that mediated by HDL alone or BSP proteins alone. The mechanism underlying the heparin-induced capacitation is unknown. Handrow et al. [51] have shown that heparin binds to bull sperm in a typical receptor-ligand interaction. Heparin stimulates the uptake of Ca^{2+} and an intracellular alkalinization of bovine sperm [52, 53]. Moreover, recent studies have shown that heparin-induced bovine sperm capacitation is associated with an increase in protein phosphorylation [54]. Further work is required to determine whether or not the BSP proteins play a role in the protein phosphorylation events that are reported to occur during capacitation.

We showed that BSP proteins interact with HDL [35]. We also showed that BSP proteins bind to choline phospholipids of the sperm membrane [32]. On the basis of these results, we previously postulated that after ejacula-

tion, BSP proteins coat the sperm membrane and that sperm-bound BSP proteins interact with HDL, resulting in sequestration of phospholipids and cholesterol. This would result in the modification of the sperm membrane lipid composition (decrease in cholesterol/phospholipid ratio) or capacitation. In view of the current results, we now propose a modified mechanism for sperm capacitation. After ejaculation of the spermatozoa and a brief exposure (about 20–30 min) to seminal fluid (BSP proteins), there is a small but significant cholesterol efflux. At the same time, sperm are coated with BSP proteins. The first cholesterol efflux induced by the BSP proteins may slightly destabilize the sperm membrane. This binding of the BSP proteins may induce a reorganization of the membrane, which could result in the appearance of new receptors (for heparin-like glycosaminoglycan or HDL) at the sperm surface. The sperm, which are now coated with BSP proteins, then travel through the female genital tract, where they may be capacitated by two different mechanisms. 1) In heparin-induced capacitation, the binding of BSP proteins to the sperm membrane increases the number of heparin-binding sites on the sperm surface. Heparin binds to these sites and induces capacitation. Indeed, bovine epididymal sperm have few binding sites for heparin and are unable to undergo capacitation within the same time period as ejaculated sperm exposed to heparin [55, 56]. However, a 20-min exposure of epididymal sperm to seminal plasma reduces the time required for heparin-induced capacitation to 4 h [56, 57]. This stimulatory effect is due to a group of heparin-binding proteins (14–18-, 24-, and 31-kDa) that are the same as BSP proteins [34, 36, 56]. 2) Alternatively, in HDL-induced capacitation, BSP protein-coated sperm could interact with HDL, which could stimulate the second sperm cholesterol efflux in the female genital tract. This may result in a further decrease in the cholesterol:phospholipid ratio, leading to capacitation. Since cholesterol is recognized to have a stabilizing effect on membranes [58], its efflux would be expected to provoke further reorganization or destabilization of the membrane. This could regulate the surface expression of sperm zona pellucida receptors [20]. The adhesion to the zona pellucida would then trigger the AR.

In summary, we have found for the first time that the BSP proteins and HDL stimulate epididymal sperm cholesterol efflux. The efflux of sperm cholesterol appears to occur at two levels. The first efflux is mediated by BSP proteins during ejaculation when sperm are exposed to seminal fluid for a brief period; the second efflux, which appears to be more significant, is mediated by HDL in the female genital tract. These two events complete sperm capacitation. Our results also indicate that the mechanisms of capacitation induced by heparin and HDL are different. Further work is underway to delineate the differences between these two mechanisms.

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