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Cholesterol Efflux-Mediated Signal Transduction in Mammalian Sperm: Cholesterol Release Signals an Increase in Protein Tyrosine Phosphorylation during Mouse Sperm Capacitation

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We previously demonstrated that mouse sperm capacitation is accompanied by a time-dependent increase in protein tyrosine phosphorylation that is dependent on the presence of BSA, Ca^{2+}, and $NaHCO_3$, all three of which are also required for this maturational event. We also demonstrated that activation of protein kinase A (PK-A) is upstream of this capacitation-associated increase in protein tyrosine phosphorylation. BSA is hypothesized to modulate capacitation through the removal of cholesterol from the sperm plasma membrane. In this report, we demonstrate that incubation of mouse sperm medium containing BSA results in a release of cholesterol from the sperm plasma membrane to the medium; release of this sterol does not occur in medium devoid of BSA. We next determined whether cholesterol release leads to changes in protein tyrosine phosphorylation. Blocking the action of BSA by adding exogenous cholesterol-SO_4^- to the BSA-containing medium inhibits the increase in protein tyrosine phosphorylation as well as capacitation. This inhibitory effect is overcome by (1) the addition of increasing concentrations of BSA at a given concentration of cholesterol-SO_4^- and (2) the addition of dibutyryl cAMP plus IBMX. High-density lipoprotein (HDL), another cholesterol binding protein, also supports the capacitation-associated increase in protein tyrosine phosphorylation through a cAMP-dependent pathway, whereas proteins that do not interact with cholesterol have no effect. HDL also supports sperm capacitation, as assessed by fertilization *in vitro*. Finally, we previously demonstrated that HCO_3^- is necessary for the capacitation-associated increase in protein tyrosine phosphorylation and demonstrate here, by examining the effectiveness of HCO_3^- or BSA addition to sperm on protein tyrosine phosphorylation, that the HCO_3^- effect is downstream of the site of BSA action. Taken together, these data demonstrate that cholesterol release is associated with the activation of a transmembrane signal transduction pathway involving PK-A and protein tyrosine phosphorylation, leading to functional maturation of the sperm.

Key Words: **mouse; sperm; capacitation; cholesterol; cAMP; protein tyrosine phosphorylation.**

INTRODUCTION

Mammalian sperm do not possess the ability to fertilize the egg immediately upon ejaculation, but acquire fertilization competence following residence in the female tract for a finite period. This time-dependent acquisition of fertilizing ability is known as "capacitation" and was first observed by both Chang (1951, 1955) and Austin (1951, 1952); capacitation can also be accomplished *in vitro* using defined media. The definition of capacitation has been modified over the years to include the acquisition of the ability of the acrosome-intact sperm to undergo the acrosome reaction in response to the *zona pellucida* (Florman and Babcock, 1991; Kopf and Gerton, 1991; Ward and Storey, 1984) or to other physiological stimuli such as progesterone (Osman *et al.*, 1989; Wistrom and Meizel, 1993).

Although capacitation can be mimicked *in vitro* by incubating epididymal or ejaculated sperm in a defined

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medium, the exact composition of media that support capacitation is variable for different species. Usually, these media represent balanced salt solutions containing appropriate concentrations of electrolytes, metabolic energy sources, Ca^{2+}, HCO_3^-, and a protein source that usually is bovine serum albumin (BSA); this composition in many instances approximates that of oviduct fluid (Yanagimachi, 1994). The function of these different medium components to support capacitation *in vitro* is not well understood at the molecular level. Work in several species has suggested that the presence of serum albumin in the medium is responsible for the removal of cholesterol from the sperm plasma membrane which is known to occur during capacitation (Davis, 1976, 1981; Davis *et al.*, 1979; Go and Wolf, 1985; Langlais and Roberts, 1985; Suzuki and Yanagimachi, 1989). It also was proposed that this loss of cholesterol accounts for the membrane fluidity changes that have been documented in many species during capacitation (Wolf *et al.*, 1986b; Yanagimachi, 1994). The consequence of cholesterol loss on sperm function, however, is poorly understood.

Capacitation has also been correlated with changes in sperm intracellular ion concentrations, metabolism, and motility (Harrison, 1996; Yanagimachi, 1994). Although these changes have been known for many years to accompany the process of capacitation, the molecular basis underlying these events is poorly understood. Remarkably, since capacitation *in vitro* can occur in the absence of any specific external stimulus, it is likely that the sperm cell can intrinsically control capacitation and that aspects of this control lie within the sperm plasma membrane. For example, changes in the properties of the plasma membrane might lead to a derepression of a set of preprogrammed cellular events ultimately necessary for the development of the capacitated state. This, however, does not preclude the importance of potential positive and/or negative modulation of capacitation *in vivo* by factors associated with the male and female reproductive tracts.

Previously we demonstrated that incubation conditions conducive to capacitation *in vitro* of cauda epididymal mouse sperm promote the tyrosine phosphorylation of multiple sperm proteins (Visconti *et al.*, 1995a). This increase in protein tyrosine phosphorylation, as well as capacitation, requires the presence of BSA, Ca^{2+}, and $NaHCO_3$ in the medium (Visconti *et al.*, 1995a). Interestingly, the capacitation-associated up-regulation of protein tyrosine phosphorylation is regulated by cAMP/protein kinase A (PK-A) (Visconti *et al.*, 1995b, 1997) through the stimulation of a tyrosine kinase and/or the inhibition of a protein tyrosine phosphatase. This unique crosstalk between PK-A and tyrosine kinase signaling pathways appears unique to sperm (Visconti *et al.*, 1998). Although the requirement of BSA for capacitation is postulated to be responsible for the removal of cholesterol from the sperm plasma membrane, it is not clear how BSA functions to increase protein tyrosine phosphorylation. Our working hypothesis is that BSA functions as a sink for cholesterol and that changes in the levels of plasma membrane cholesterol lead to changes in the fluidity of the plasma membrane and a subsequent increase in the permeability of the sperm to Ca^{2+} and HCO_3^- (Yanagimachi, 1994). The resultant increases in the levels of Ca^{2+} and HCO_3^- would ultimately stimulate the activity of the sperm adenylyl cyclase, leading to an increase in cAMP and an increase in protein tyrosine phosphorylation (Visconti *et al.*, 1995b, 1997).

In the present study, we set out to determine whether the effect of BSA on protein tyrosine phosphorylation and capacitation in mouse sperm involved cholesterol removal. We demonstrate that cholesterol was released into the medium from sperm only when these cells were incubated in the presence of BSA. Abrogation of cholesterol binding to BSA by addition of cholesterol-SO_4^- to the medium blocks both the increase in protein tyrosine phosphorylation and inhibits capacitation. As predicted, increasing concentrations of BSA, as well as cAMP agonists, overcame this cholesterol-SO_4^- inhibition on protein tyrosine phosphorylation. Another cholesterol binding protein, namely high-density lipoprotein (HDL), replaced BSA in media to promote protein tyrosine phosphorylation and fertilization *in vitro*. On the other hand proteins that do not bind cholesterol are not able to increase protein tyrosine phosphorylation. These data, taken together, demonstrate that cholesterol removal from the plasma membrane is linked to a unique signal transduction pathway that involves crosstalk between PK-A and tyrosine kinase signaling pathways leading to protein tyrosine phosphorylation and functional maturation of the sperm.

MATERIALS AND METHODS

Materials

Cholesterol and cholesterol-SO_4^-, BSA (Fraction V; fatty acid poor), desmosterol, filipin, pregnenolone-SO_4^-, androstenedione-SO_4^-, cholesterol oxidase (C-8153), protease inhibitors, ovalbumin, cytochrome *c*, histone H2A, myelin basic protein, and kemptide were obtained from Sigma. HDL that had been purified according to Pomerantz and Hajjar (1990) was a gift from Ken Pomerantz, Cornell Medical College. Anti-phosphotyrosine antibody (clone 4G10) was from UBI (Lake Placid, NY). Organic solvents were from EM Sciences (chromatographic grade). Unibond RP_{18}/silica gel diphasic plates (10 × 12 cm, 250-μm thickness) were obtained from Analtech, Inc. (Newark, DE). HP-K silica gel plates (10 × 10 cm, 250-μm thickness) and P-81 phosphocellulose papers were purchased from Whatman, Inc. (Clifton, NJ). [γ-^{32}P]ATP (3000 Ci/mmol) and ECL reagents were from Amersham. Immobilon P was purchased from Millipore (Bedford, MA) and EcoLite scintillation fluid was from ICN (Costa Mesa, CA).

Culture Media

The basic medium used throughout these studies for the preparation of sperm was a modified Krebs–Ringer bicarbonate medium (HMB-Hepes buffered), as described by Lee and Storey (1986). This medium was first prepared in the absence of Ca^{2+}, BSA, and pyruvate, sterilized by passage through a 0.20-μm filter (Nalgene), and frozen at −20°C in aliquots for single use. Working "complete" media were prepared by adding Ca^{2+} (1.7 mM), pyruvate (1 mM),

and BSA (3 mg/ml), followed by gassing with 5% CO_2 in air to pH 7.3. HM medium was derived from HMB by replacing the $NaHCO_3$ with NaCl, while maintaining the same pH. In some experiments, the concentrations of BSA or $NaHCO_3$ were adjusted, and the pH was maintained at 7.3.

Preparation of Sperm

Caudal epididymal mouse sperm were collected from CD1 retired breeder males by placing one minced cauda epididymis in 0.5 ml of medium HM without Ca^{2+} or BSA. After 5 min the sperm suspension was washed in 10 ml of the same medium by centrifugation at 800*g* for 10 min at room temperature (24°C). The sperm were resuspended to a final concentration of 5–10 $\times$ 10^7 cells/ml and diluted 10 times in the appropriate medium depending on the experiment performed. After incubation for 1.5 h, except where indicated, the sperm were concentrated by centrifugation at 5000*g* for 1 min (24°C), and the sperm pellet was washed in 1 ml of phosphate-buffered saline (PBS), resuspended in sample buffer (Laemmli, 1970) without 2-mercaptoethanol, and boiled for 5 min. After centrifugation at 5000*g* for 3 min, the supernatant was removed, 2-mercaptoethanol was added to a final concentration of 5%, and the samples were boiled for 5 min and then subjected to SDS–PAGE as described below.

Solubilization of Steroids

All of the steroid sulfates were solubilized using DMSO to a final stock concentration of 100 mM. In contrast, cholesterol was directly added to the incubation medium to the appropriate concentration and the solution then sonicated. The final concentration of DMSO was never higher than 0.5%; this concentration has no effect on capacitation or the increase in protein tyrosine phosphorylation. The standard solutions of the different steroid–sulfates used in these experiments were diluted to various final concentrations as stated in the designated experiment, added to the medium containing BSA, and preincubated for 30 min prior to the addition of the sperm suspension.

Steroid Measurements

Sperm (5 $\times$ 10^6 cells) were incubated for 1.5 h in 0.5 ml of medium in the absence or presence of 3 mg/ml BSA. After this period, each sample was centrifuged (10,000*g;* 10 min) and the resultant sperm pellets and supernatants were assayed for sterol content as described previously (Alvarez and Storey, 1995). Briefly, sperm pellets were extracted with 20 vol of chloroform:methanol (1:1, v/v) and sperm supernatants were extracted with 6 vol of chloroform:methanol (2:1, v/v). The samples were then vortexed (10 s) and centrifuged at 800*g* (3 min) and the resultant organic phases evaporated to dryness under N_2. The evaporated organic phases were then dissolved in 20 μl of chloroform:methanol (1:1, v/v) and aliquots of 4 μl applied to silver nitrate-impregnated Whatman HP-K silica gel microplates. Aliquots (4 μl) of cholesterol, desmosterol, cholesterol sulfate, and desmosterol standards were applied on separate lanes as reference standards. The plates were predeveloped in chloroform:methanol (1:1, v/v) to 1 cm from the lower edge of the plate. This predevelopment step is used to minimize eddy diffusion that results in band broadening and lower resolution. Following predevelopment, the plates were thoroughly dried and then developed in chloroform:acetone (95:5, v/v) in the same dimension. Following development, the plates were thoroughly dried, dipped in a 10% solution of copper sulfate in 8% phosphoric acid, and placed on a CAMAG Plate Heater III at 185°C for 5 min. The resulting bands were scanned at 400 nm in the reflectance mode using a Shimadzu CS-9000 spectrodensitometer (Shimadzu Scientific Instruments, Columbia, MD). The integrated areas obtained for the unknowns were interpolated with the standard curves obtained for the respective cholesterol, desmosterol, and cholesterol sulfate standard curves, and the values were expressed as nanograms per 10^6 cells.

To control for nonspecific release of cholesterol from the sperm due to the centrifugation step itself under the different incubation conditions (±BSA), the following controls were performed. Sperm suspensions were incubated in medium in either the absence or the presence of BSA as described above but, prior to centrifugation, 50 μl of a 4 mg/ml solution of cholesterol oxidase in an isotonic buffer (pH 7.4) was added to 500 μl of the sperm suspension (5 $\times$ 10^6 sperm). The suspension was incubated at 24°C for 10 min. Enzyme reactions were terminated by the addition of 50 μl of 3 N HCl and the tubes were then vortexed for 5 s. Following centrifugation at 10,000*g* for 10 min, the sperm pellet and the sperm-free supernatant were assayed for the presence of cholesterol and 4-cholestene-3-one (the product of cholesterol oxidation) (Alvarez and Storey, 1995).

Identification of Filipin–Cholesterol Complexes in Mouse Sperm

Cauda epididymal sperm were obtained as described above. Aliquots of 10^6 sperm were then incubated in HMB medium in the absence or presence of 3 mg/ml BSA for 1.5 h. After this incubation period, sperm were analyzed for the presence of plasma membrane cholesterol by monitoring the formation of filipin–sterol complexes by freeze–fracture electron microscopy as previously described (Diaz-Fontdevila *et al.*, 1992). Briefly, the sperm suspensions were centrifuged at 500*g* for 10 min and washed twice with PBS. The final sperm pellet was resuspended in a fixative solution containing 3% glutaraldehyde in 0.1% cacodylate buffer, pH 7.4, in the presence of 0.02% filipin for 1 h at 4°C. Filipin was initially solubilized in DMSO as a 1% solution. An aliquot of sperm was also fixed in the absence of filipin and served as a control. Aliquots (500 μl) of fixed sperm were centrifuged in a microfuge at maximum speed for 1 min and the sperm pellets were resuspended and incubated in 500 μl of 25% glycerol in PBS. After 2 h, the suspensions were centrifuged and the pellets mounted on gold supports, precooled in supercooled liquid N_2, and fractured using a double replica device in a Balzers BAF-301 apparatus with a stage temperature of −100°C. The specimens were coated with platinum (3–5 nm) and carbon (25 nm). The surface replicas were cleaned with Clorox and washed in double-distilled water. Specimens were then observed using a Siemens Elmiskop I at 80 kV.

SDS–PAGE and Immunoblotting

SDS–PAGE (Laemmli, 1970) was performed in 10% gels. Electrophoretic transfer of proteins to Immobilon P in all experiments was carried out according to the method of Towbin *et al.* (1979) at 70 V (constant) for 2 h at 4°C. Immunodetection was carried out at room temperature as described previously (Kalab *et al.*, 1994) using the 4G10 monoclonal antibody against anti-phosphotyrosine and enhanced chemiluminescence detection using an ECL kit according to the manufacturer's instructions. Where appropriate, changes in protein tyrosine phosphorylation were quantified by densito-

metric integration of the band of interest in the scanned film image using the NIH Image software.

Assay of Protein Kinase A Activity

Protein kinase A activity was measured as previously described (Visconti *et al.*, 1997) using kemptide as a specific peptide substrate. Briefly, sperm were adjusted to a final concentration of 10^7 cells/ml and incubated for various periods of time either in the absence or in the presence of 3 mg/ml BSA or 1 mg/ml HDL. At the appropriate time points, 10 μl of the sperm suspension was added to 10 μl of 2× assay cocktail. The final concentrations of the components in the assay cocktail after sperm addition were 100 μM kemptide, [γ-^{32}P]ATP (3000 Ci/mmol) (3×10^6 cpm/assay), 100 μM ATP, 1% (v/v) Triton X-100, 1 mg/ml BSA, 10 mM $MgCl_2$, 40 mM β-glycerophosphate, 5 mM *p*-nitrophenyl phosphate, 10 mM Tris–HCl, pH 7.4, 10 μM aprotinin, and 10 μM leupeptin. Note that the BSA present in the assay cocktail was added for the purposes of coprecipitation of the proteins following the enzymatic assay of PK-A. The samples then were incubated for 5 min at 37°C; the assay was linear at this time point using a variety of sperm protein concentrations. The reactions were stopped by adding 20 μl 20% TCA, cooled on ice for 20 min, and centrifuged at room temperature for 3 min at 10,000*g*. Thirty microliters of the resultant supernatant was then spotted onto 2 × 2-cm Whatman P81 phosphocellulose papers. The phosphocellulose papers were washed 5× 5 min in 5 mM phosphoric acid with agitation, dried, placed in vials with 2.5 ml of EcoLite scintillation cocktail, and subjected to liquid scintillation counting.

Coomassie Blue Assay for the Acrosome Reaction

As an endpoint of capacitation, we analyzed the zona pellucida (ZP)-induced acrosome reaction, based on the premise that only capacitated sperm undergo exocytosis in response to the ZP. The percentage of acrosome reactions was measured using Coomassie Blue G250 as described by Thaler and Cardullo (1995). Briefly, sperm were incubated under the desired experimental conditions for 1.5 h, followed by the addition of 5 ZP/μl or buffer, and the sperm were then incubated for an additional 30 min. ZP were isolated and solubilized as previously described (Visconti *et al.*, 1995a). An equal volume of 2× fixative solution (7.5% formaldehyde in 10 mM PBS) was then added to each tube. After 10 min, the sperm were centrifuged for 2 min at 10,000*g* and resuspended in 0.1 M ammonium acetate, pH 9. After centrifugation, the sperm pellet was resuspended in 20 to 50 μl of the same buffer, spread onto poly-L-lysine-coated slides, and air dried. The slides were then stained with Coomassie Blue G250 [0.04% (w/v) in 3.5% (v/v) perchloric acid]. After 10 min, the slides were gently rinsed with deionized H_2O until the slides appeared blue; the slides were then air dried and mounted with Permount. To calculate the percentage of acrosome reactions at least 200 sperm were counted per experimental condition. The data presented are the average of at least three individual experiments.

In Vitro Fertilization

In vitro fertilization of metaphase II-arrested mouse eggs was performed as previously described (Visconti *et al.*, 1995a). Polyvinyl alcohol (0.01%) was used to maintain the isotonic conditions when sperm were cultured in media devoid of BSA prior to insemination. When HDL was included at different concentrations in the capacitation media, the medium containing the capacitated sperm was diluted 100-fold prior to insemination of the eggs. Sperm were capacitated in the different media for a period of 2 h and then incubated with the eggs for 3 h. When HDL was present in the capacitation media, HDL was also present in the insemination media. All *in vitro* fertilizations were performed using metaphase-arrested eggs retrieved at approximately 14 h post-hCG injection. Phase-contrast optics were used to evaluate fertilization by looking for the presence of the second polar body and the formation of both the male and the female pronuclei.

Statistical Analyses

Statistical analyses were performed using a Student *t* test as described by Zar (1996).

RESULTS

Cholesterol Is Released from the Sperm Plasma Membrane under Conditions Conducive to Capacitation

Previously we demonstrated in mouse sperm that both protein tyrosine phosphorylation and capacitation required the presence of BSA in the incubation medium. The role of BSA in capacitation has been postulated to involve the removal of cholesterol from the sperm membrane (see Introduction). To determine if BSA, indeed, mediated cholesterol removal and led to the capacitation-associated increases in tyrosine phosphorylation, we first determined whether BSA was involved in the release of cholesterol from the sperm plasma membrane under our experimental conditions. Two different approaches were used to test this hypothesis. First, we directly measured the cholesterol released into the incubation medium, as well as that remaining in the sperm under conditions that do or do not support capacitation. Second, we visualized the cholesterol remaining in the plasma membrane of sperm incubated in the absence or the presence of BSA, using the polyene antibiotic filipin.

Cauda epididymal sperm were incubated in medium HMB in the presence or in the absence of 3 mg/ml BSA. After 1.5 h of incubation, cholesterol released to the incubation medium as well as the cholesterol remaining with the sperm under these two experimental conditions was determined. A significant increase in medium cholesterol was observed in sperm suspensions incubated in medium containing BSA compared to sperm suspensions incubated in medium devoid of BSA (Table 1), suggesting that medium that supports capacitation promotes the release of sperm-associated cholesterol. This observation was correlated with a decrease in the cholesterol associated with the resultant sperm pellets. Incubation of sperm in medium containing BSA also increased the release of desmosterol into the incubation medium, but did not affect the release of cholesterol-SO_4^- to a measurable extent (Table 1).

In order to rule out the possibility that the release of cholesterol from sperm incubated in medium containing BSA was due, either in part or completely, to a nonspecific

TABLE 1

BSA-Induced Release of Cholesterol, Desmosterol, and Cholesterol-SO_4^- from Mouse Sperm

		Steroids[a,b]		
Treatment	Fraction	C	D	CSO_4^-
−BSA	Medium	5 ± 1.6	3 ± 1	ND
	Sperm	416 ± 21	277 ± 12	19 ± 3
+BSA	Medium	59 ± 5.6*	43 ± 3.3*	ND
	Sperm	363 ± 19**	238 ± 14**	17 ± 2.7

[a] Mean ± SEM ($n = 10$) sterol content (ng/10^6 cells) for sperm and supernatant fractions after a 1.5-h incubation in the absence or presence of BSA (3 mg/ml). Media not containing sperm had no detectable sterol content (data not shown).

[b] Abbreviations used: C, cholesterol; D, desmosterol; CSO_4^-, cholesterol sulfate; ND, not detectable. For incubations in medium containing BSA, sperm contain less (**$P < 0.01$) and the medium contains more (*$P < 0.0005$) cholesterol and desmosterol, compared to incubations in medium devoid of BSA.

release as a consequence of the centrifugation process, cholesterol oxidase was added to the sperm suspensions after the incubation but prior to the centrifugation (see Materials and Methods). The enzyme was then inactivated prior to centrifugation. Membrane-bound cholesterol would not serve as a substrate for this enzyme but, in contrast, any cholesterol released during the incubation period would be quantitatively converted to cholestene-3-one. Under these conditions, any cholesterol subsequently released during the centrifugation step and after cholesterol oxidase inactivation would be measured as cholesterol. Using this experimental approach, all of the cholesterol released was in the form of cholestene-3-one (50 ± 3.2 ng sterol/10^6 cells) and the cholesterol remaining associated with the sperm remained as cholesterol (270 ± 24 ng sterol/10^6 cells). In contrast when the sperm were incubated in media devoid of BSA neither cholestene-3-one nor cholesterol was found in the medium and all of the sperm-associated sterol was in the form of cholesterol (320 ± 23 ng sterol/10^6 cells). No cholesterol-3-one was detected when cholesterol oxidase was not added (data not shown). These data demonstrate that the cholesterol released into the medium during the incubation does not occur as a consequence of the centrifugation step.

Changes in the cholesterol content of the sperm plasma membrane were also monitored by exploiting the cholesterol-binding properties of the polyene antibiotic filipin (Diaz-Fontdevila *et al.*, 1992; Lin and Kan, 1996; Suzuki, 1988; Suzuki and Yanagimachi, 1989; Toshimori *et al.*, 1985). Using freeze–fracture electron microscopic analysis, the cholesterol–filipin complexes were visualized as spherical particles (Fig. 1). In contrast to sperm incubated in medium devoid of BSA (Fig. 1A), the density and number of cholesterol–filipin complexes were significantly lower in sperm incubated in the presence of BSA (Fig. 1B). In these experiments, approximately 80% of the cells examined under noncapacitating conditions (i.e., −BSA) displayed a pattern similar to that seen in Fig. 1A. In contrast, approximately 90% of the cells examined under capacitating conditions (i.e., +BSA) displayed a pattern similar to that seen

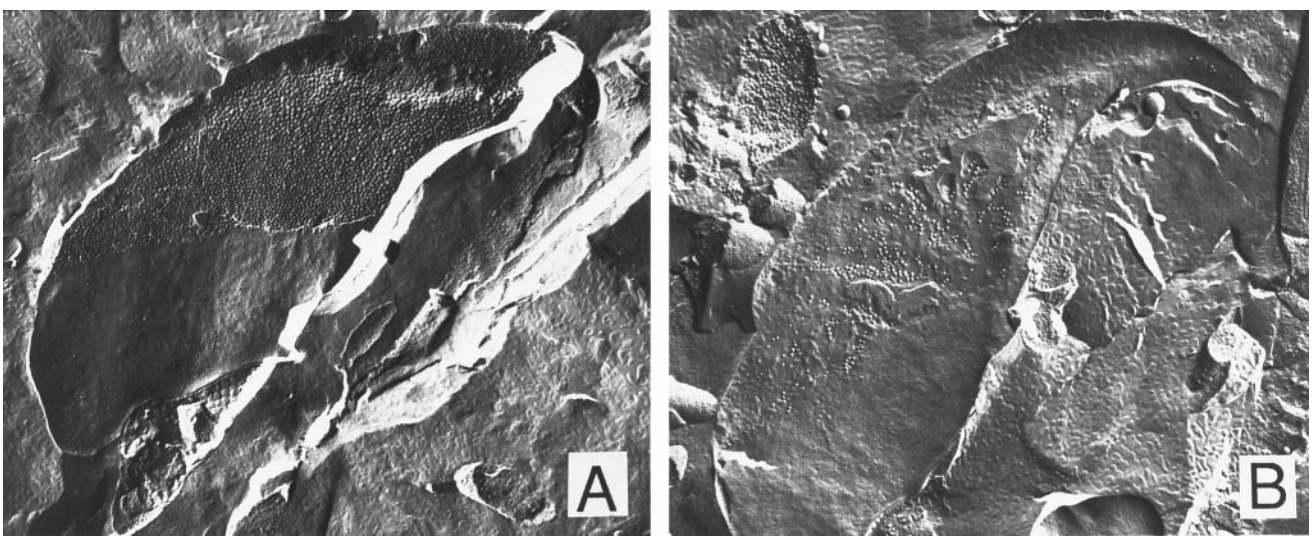

FIG. 1. Identification of filipin–cholesterol complexes in mouse sperm. Sperm were incubated in HMB medium either in the absence (A) or in the presence (B) of 3 mg/ml BSA for 1.5 h. After this incubation period, sperm were analyzed for the presence of plasma membrane cholesterol by monitoring the formation of filipin–cholesterol complexes by freeze–fracture electron microscopy as described under Materials and Methods. Note that these complexes appear as spherical structures in freeze fracture. The experiment was performed three times with similar results.

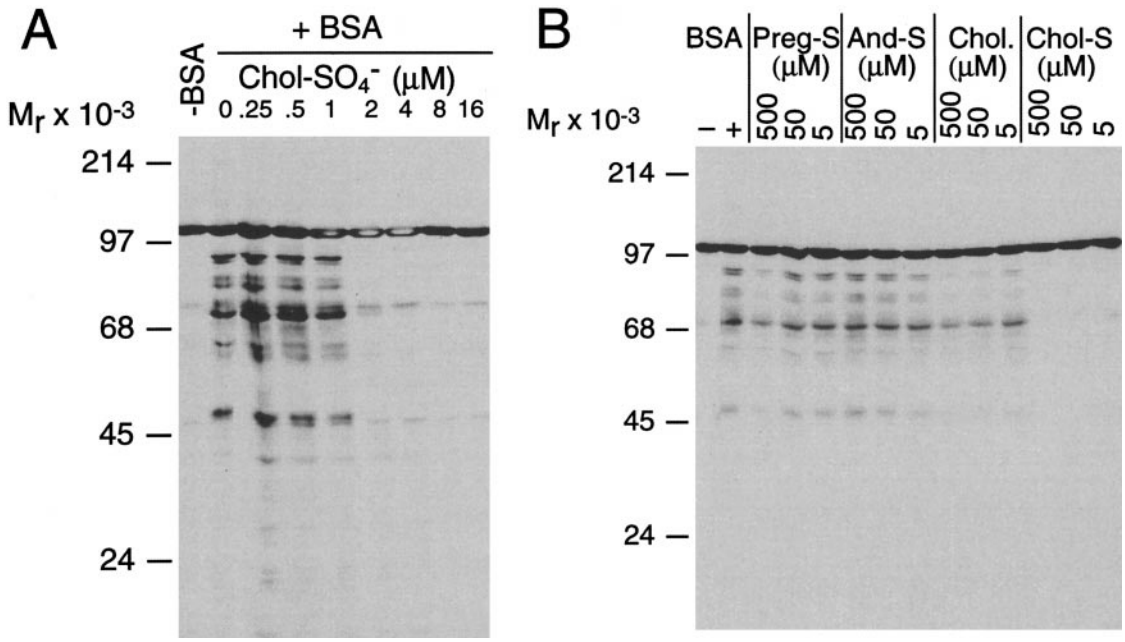

FIG. 2. Effects of various sterols and sterol sulfates on the capacitation-associated increase in protein tyrosine phosphorylation. (A) Concentration-dependent effects of cholesterol-SO_4. Cauda epididymal mouse sperm were collected as described under Materials and Methods and incubated in HMB medium in the absence (−BSA) or in the presence of 3 mg/ml BSA and in the presence of increasing concentrations of cholesterol-SO_4^- as shown. After 1.5 h of incubation the pattern of protein tyrosine phosphorylation was analyzed by PAGE and immunoblotted using anti-phosphotyrosine antibodies. (B) Specificity of steroid inhibition. Cauda epididymal sperm were incubated for 1.5 h in the absence or in the presence of 3 mg/ml BSA and in the presence of increasing concentrations of different steroids or steroid sulfates as shown. The sperm were then analyzed by PAGE and immunoblotted using anti-phosphotyrosine antibodies. These experiments were performed at least three times with similar results; shown are representative experiments.

in Fig. 1B. Although the decrease in cholesterol–filipin complexes is obvious in the head region of the sperm (Fig. 1), it was also possible to observe a decrease in cholesterol in the plasma membrane surrounding the principal piece of the sperm (data not shown). Taken together, these experiments demonstrate that there is a release of cholesterol from the plasma membrane surrounding the sperm anterior head and tail, and the data are consistent with previous reports of cholesterol release in sperm (Suzuki and Yanagimachi, 1989) as well as modifications in the properties of the plasma membrane that are observed during capacitation (Wolf and Cardullo, 1991). These observations also raise the possibility that changes in plasma membrane cholesterol could affect other processes during capacitation related to flagellar movement, such as hyperactivation of motility.

Inhibition of Protein Tyrosine Phosphorylation and Capacitation by Cholesterol-SO_4^-

To investigate whether the effect of BSA on the capacitation-related changes in protein tyrosine phosphorylation and on capacitation was due to cholesterol removal from the plasma membrane, we determined whether these two aforementioned parameters were affected by the addition to the media of increasing concentrations of a soluble cholesterol analogue such as cholesterol-SO_4^-. The rationale behind these experiments is that if BSA normally serves as an acceptor for plasma membrane cholesterol, addition of a cholesterol analog should increase the total sterol content in the capacitation media and render BSA unable to capacitate the sperm. When sperm were incubated in medium containing 3 mg/ml BSA plus increasing concentrations of cholesterol-SO_4^-, the capacitation-associated increase in protein tyrosine phosphorylation was inhibited by this steroid sulfate with an IC_{50} of between 1 and 2 μM (Fig. 2A). It should be noted that in Fig. 2A and in all subsequent figures, the major phosphotyrosine-containing protein at M_r 116,000 whose phosphorylation is capacitation-independent is an isoform of hexokinase type I with unique properties (Kalab *et al.*, 1994).

The cholesterol-SO_4^- inhibition of the capacitation-related increase in protein tyrosine phosphorylation was specific to the cholesterol moiety of the molecule, since sperm incubated in medium containing BSA and different concentrations of androstenedione-SO_4^- or pregnenolone-SO_4^- displayed protein tyrosine phosphorylation patterns similar to those of sperm incubated in the control medium (Fig. 2B). Cholesterol, although inhibitory, was not as effective as cholesterol-SO_4^- in inhibiting protein tyrosine phos-

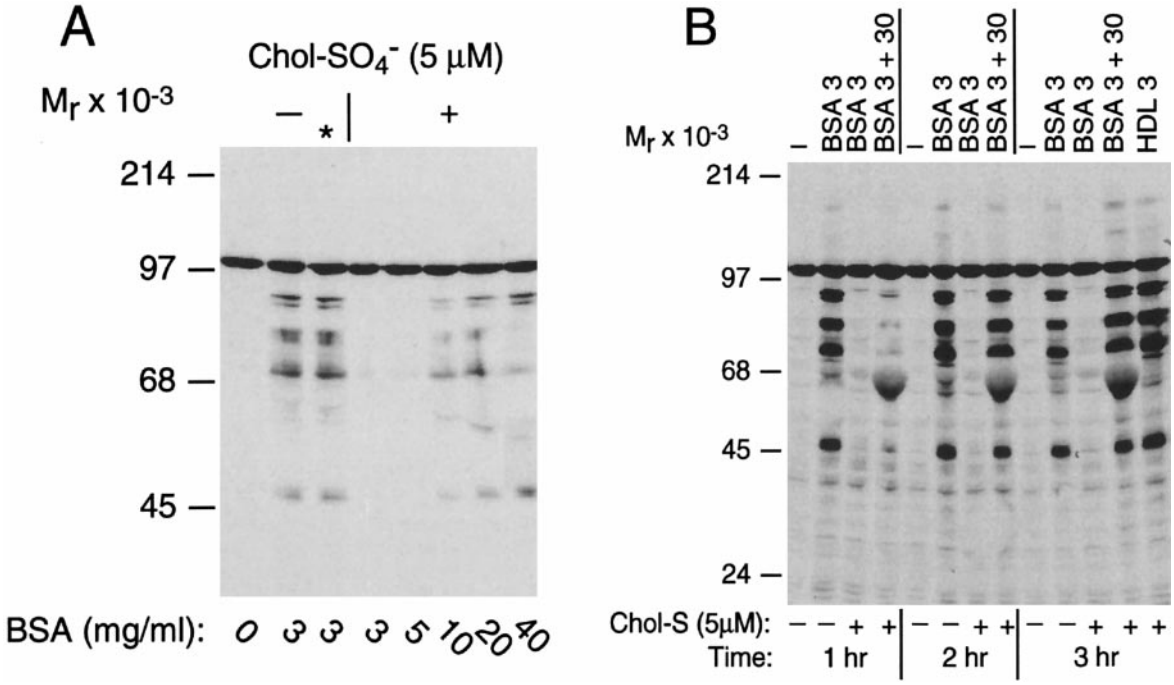

FIG. 3. Effect of increasing concentrations of BSA on the cholesterol-SO_4^- inhibition of capacitation-associated protein tyrosine phosphorylation. (A) Cauda epididymal sperm were collected as described under Materials and Methods and incubated in HMB medium for 1.5 h in the absence or in the presence of increasing concentrations of BSA and/or 5 μM cholesterol-SO_4^- as shown. The asterisk represents a control with 3 mg/ml BSA and 0.5% DMSO. (B) Cauda epididymal sperm were collected as described under Materials and Methods and incubated in the absence or in the presence of 3 mg/ml BSA and/or 5 μM cholesterol-SO_4^- as shown. After 1.5 h, BSA was added to a final concentration of 30 mg/ml to some of the sperm suspensions and the sperm were incubated for an additional 1, 2, and 3 h. A similar experiment was performed using HDL and is shown. In both experiments, the pattern of protein tyrosine phosphorylation in the different samples was analyzed by PAGE and immunoblotting using anti-phosphotyrosine antibodies. The experiments were performed at least three times with similar results. Shown are representative experiments.

phorylation, likely due to the limited solubility of this steroid in the capacitation medium.

If the effects of cholesterol-SO_4^- were due to the inhibition of BSA action leading to an increase in protein tyrosine phosphorylation, addition of higher concentrations of BSA to the incubation medium containing a fixed concentration of cholesterol-SO_4^- should bypass the inhibitory effect of cholesterol-SO_4^- on protein tyrosine phosphorylation, and this was observed to be the case (Fig. 3A). Since this experiment was performed by incubating the sperm in the presence of greater concentrations of BSA using a fixed concentration of cholesterol-SO_4^- (5 μM), the ability of BSA to overcome the cholesterol-SO_4^- inhibition of protein tyrosine phosphorylation could be explained in two ways. First, if the concentration of BSA were increased, thereby increasing the effective concentration of cholesterol binding sites available in the medium, BSA would no longer be limiting in terms of its ability to sequester sperm cholesterol and would therefore support protein tyrosine phosphorylation. Alternatively, the addition of increasing concentrations of BSA would quench the available cholesterol-SO_4^- in the medium and in this way would block the inhibitory action of cholesterol-SO_4^-. To distinguish between these two possibilities, we incubated the sperm in medium containing 3 mg/ml BSA and 5 μM cholesterol-SO_4^- for 1 h, which inhibits protein tyrosine phosphorylation (as shown in Figs. 2 and 3). After 1 h incubation, additional BSA was added to a final concentration of 30 mg/ml, and the sperm were further incubated for various periods of time. The addition of BSA bypassed the inhibitory effect of cholesterol-SO_4^- (Fig. 3B). Although these data do not demonstrate that cholesterol-SO_4^- is blocking the release of cholesterol from the sperm, they demonstrate: (1) that the inhibition by cholesterol-SO_4^- is reversible and can be overcome with increasing concentrations of BSA and (2) that cholesterol-SO_4^- is not irreversibly toxic to the sperm.

We previously demonstrated that a cAMP pathway that was downstream of the action of BSA, Ca^{2+}, and $NaHCO_3$ mediated the capacitation-associated changes in protein tyrosine phosphorylation (Visconti *et al.*, 1995b). The evidence supporting this conclusion is based on the use of two inhibitors of PK-A activity (i.e., H-89 and RpcAMPS) which inhibit the enzyme by totally different mechanisms (Visconti *et al.*, 1995b). To further investigate whether the action of BSA on protein tyrosine phosphorylation is mediated by a cAMP/PK-A pathway, we measured PK-A activity

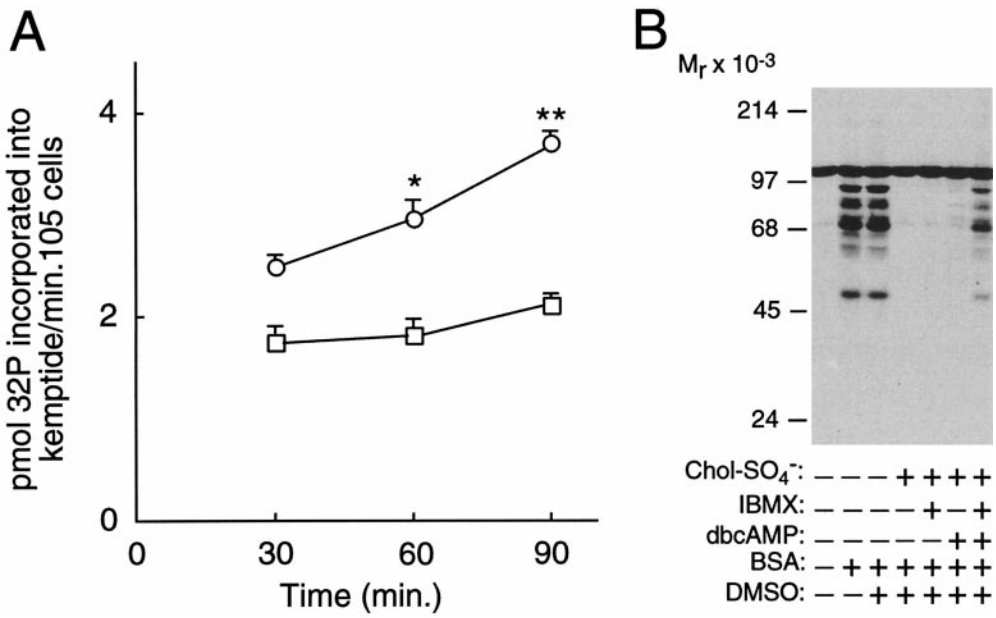

FIG. 4. Role of cAMP and protein kinase A in mediating the BSA-induced changes in protein tyrosine phosphorylation of mouse sperm. (A) BSA-induced changes in PK-A activity during mouse sperm capacitation. Caudal epididymal sperm were incubated in HMB medium in the absence (□) or in the presence (○) of 3 mg/ml BSA for the time periods indicated on the abscissa. PK-A activity was then assayed as described under Materials and Methods. This experiment was performed three times in triplicate, and the values shown represent the means ± SEM ($n = 9$; $^*P < 0.05$; $^{**}P < 0.01$ from the same time in the respective −BSA control). (B) Effects of dibutyryl cAMP and IBMX on the cholesterol-SO_4^- inhibition of the capacitation-associated increase in protein tyrosine phosphorylation. Cauda epididymal sperm were collected as described under Materials and Methods and incubated in HMB medium for 1.5 h in the absence or in the presence of 3 mg/ml BSA and in some cases 5 μM cholesterol-SO_4^-, as indicated. Some of the treatments were also supplemented with 1 mM dibutyryl cAMP and 100 μM IBMX as indicated. The pattern of protein tyrosine phosphorylation in the different samples was analyzed by PAGE and immunoblotted using anti-phosphotyrosine antibodies. This experiment was performed at least three times with similar results, and a representative experiment is shown.

in media containing or devoid of 3 mg/ml BSA at different incubation times. PK-A activity is significantly higher when sperm are incubated in BSA-containing media compared to BSA-free media (Fig. 4A). Moreover, if the cholesterol-SO_4^- effects on protein tyrosine phosphorylation were due to an abrogation of BSA function, cAMP agonists should overcome the inhibitory effect of cholesterol-SO_4^- on protein tyrosine phosphorylation. As predicted, addition of 1 mM dibutyryl cAMP plus 100 μM IBMX, an inhibitor of cyclic nucleotide phosphodiesterases, induced an increase in protein tyrosine phosphorylation in medium containing BSA plus 5 μM cholesterol-SO_4^- (Fig. 4B).

To further analyze the correlation between the increase in protein tyrosine phosphorylation and capacitation, we investigated the action of cholesterol-SO_4^- on a biological endpoint of capacitation, namely the spontaneous and ZP-induced acrosome reactions (Florman and Babcock, 1991; Visconti *et al.*, 1998; Yanagimachi, 1994). Sperm were incubated for 1 h in complete medium (3 mg/ml BSA) in the presence or the absence of 5 μM cholesterol-SO_4^-. After this period, buffer or solubilized ZP (5 ZP/μl) were added, the sperm were incubated for an additional 30 min, and the percentage acrosome reactions was assessed as described under Materials and Methods. Cholesterol-SO_4^- inhibited the spontaneous acrosome reaction to levels similar to that observed in medium devoid of BSA (Fig. 5). Moreover, sperm incubated in the presence of cholesterol-SO_4^- did not undergo the acrosome reaction following treatment with ZP (Fig. 5), the physiological inducer of the acrosome reaction, demonstrating that cholesterol-SO_4^- treatment inhibited capacitation.

Promotion of Protein Tyrosine Phosphorylation and Capacitation by HDL

HDL is a complex of lipids and proteins that mediates cholesterol transfer *in vivo*. If BSA functions to increase sperm protein tyrosine phosphorylation and capacitation as a consequence of its ability to bind cholesterol, one would expect HDL to substitute for BSA in promoting these posttranslational modifications and capacitation. In contrast, proteins that do not bind cholesterol should not be able to increase protein tyrosine phosphorylation. To test this hypothesis, sperm were incubated for 1.5 h in HMB medium devoid of BSA but containing 3 mg/ml each of HDL, ovalbumin, cytochrome *c*, histone H2A, and myelin

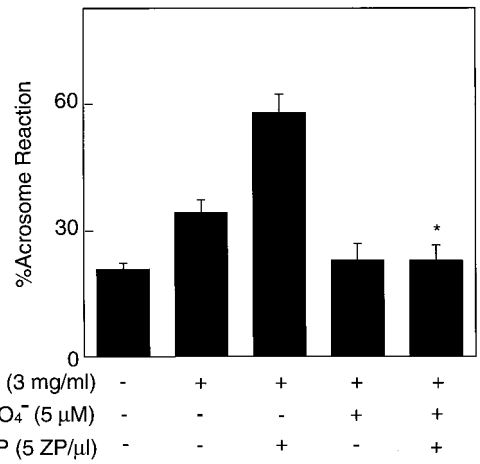

FIG. 5. Effect of cholesterol-SO_4^- on the percentage of sperm undergoing spontaneous and zona pellucida-induced acrosome reactions. Cauda epididymal sperm were collected as described under Materials and Methods and incubated in HMB medium in the absence or in the presence of 3 mg/ml BSA and in some cases of 5 μM cholesterol-SO_4^-. After a 1-h incubation, solubilized ZP were added where indicated to a final concentration of 5 ZP/μl. After an additional 30 min of incubation, the status of the acrosome was monitored using the Coomassie blue method as described under Materials and Methods. Data represent the means ± SEM, $n = 5$. Cholesterol-SO_4^- inhibited significantly the ZP-induced acrosome reaction ($^*P < 0.01$), compared to the respective ZP control in the absence of cholesterol-SO_4^-.

basic protein. As shown in Fig. 6A, BSA and HDL were the only proteins that supported protein tyrosine phosphorylation, consistent with their abilities to bind cholesterol. HDL increased protein tyrosine phosphorylation in a concentration-dependent manner with a maximum effect at 1 mg/ml (Fig. 6B). Moreover, the PK-A inhibitor H-89 blocked the effect of HDL on protein tyrosine phosphorylation (Fig. 6C), suggesting that the signaling pathway activated by HDL-induced cholesterol release from the sperm membrane leads to a PK-A up-regulation of protein tyrosine phosphorylation, similar to that seen with BSA (Visconti *et al.*, 1995b). Effects of HDL on sperm PK-A activity were confirmed by measuring the activity of this enzyme in media containing HDL as was performed with BSA in Fig. 4A. HDL-containing media supported an increase in PK-A activity compared to media devoid of this protein (data not shown), and these effects were similar to those observed with BSA-containing media (Fig. 4A). Moreover, as shown in Fig. 3B, HDL was observed to overcome the inhibition of protein tyrosine phosphorylation by cholesterol-SO_4^-, similar to that observed with BSA. Finally, HDL supported sperm capacitation as demonstrated by its ability to replace BSA in an *in vitro* fertilization assay (Fig. 6D). Heparin, which has been demonstrated to support protein tyrosine phosphorylation and capacitation in bovine sperm (Galantino-Homer *et al.*, 1997), did not support these posttranslational modifications in mouse sperm (Fig. 6A), consistent with the inability of heparin to support capacitation in this species.

Recovery of Protein Tyrosine Phosphorylation after Incubation of Sperm in Media Lacking $NaHCO_3$ or BSA

Previously we demonstrated that mouse sperm incubated in the absence of $NaHCO_3$ or in the absence of BSA did not undergo the capacitation-dependent changes in protein tyrosine phosphorylation or capacitation (Visconti *et al.*, 1995a). It is known that following incubation in medium lacking BSA or $NaHCO_3$ for 1 h, the sperm can recover their ability to be capacitated if the component lacking is added back to the medium (Neill and Olds-Clarke, 1987). To determine if this recovery was correlated with an increase in protein tyrosine phosphorylation, sperm were incubated in the absence of BSA for 1 h followed by the addition of BSA to a final concentration of 3 mg/ml. Under these conditions, the sperm undergo capacitation and protein tyrosine phosphorylation after the additional 90 min of incubation in this new medium (data not shown). A similar result was observed when the sperm were incubated in the absence of $NaHCO_3$ for 1 h followed by the addition back to the medium of 10 mM $NaHCO_3$ (data not shown).

Since sperm incubated in the absence of $NaHCO_3$ or BSA did not display an increase in protein tyrosine phosphorylation, we chose to investigate the effect of reintroducing these medium components on the kinetics of phosphorylation. The aim of these experiments was to elucidate the hierarchy of BSA and $NaHCO_3$ in this signal transduction cascade. After adding back BSA, the increase in protein tyrosine phosphorylation is slow (Fig. 7A, right) with kinetics similar to the increase in phosphorylation generally observed when noncapacitated sperm are incubated directly in a complete medium that would support capacitation (Visconti *et al.*, 1995a). In contrast, the effect of adding back $NaHCO_3$ on protein tyrosine phosphorylation is significantly more rapid than that seen with the BSA (Fig. 7A, left). To compare the kinetics of these effects, we quantified the extent of protein tyrosine phosphorylation of a M_r 95,000 protein (position of protein designated by the small dot between the two blots in Fig. 7A) as a marker for the pattern of protein tyrosine phosphorylation (Fig. 7B). These data clearly demonstrate that the $NaHCO_3$ effect on protein tyrosine phosphorylation is more rapid and suggest that the target of BSA action may be the rate-limiting step in the initiation of signaling leading to protein tyrosine phosphorylation. This is supported by the observation that increasing concentrations of $NaHCO_3$ added to medium devoid of BSA can overcome the inability of BSA-free medium to support protein tyrosine phosphorylation (Fig. 7C, left), whereas the opposite is not true (Fig. 7C, right).

DISCUSSION

We previously demonstrated that mouse, bull, and human sperm capacitation *in vitro* is tightly correlated with

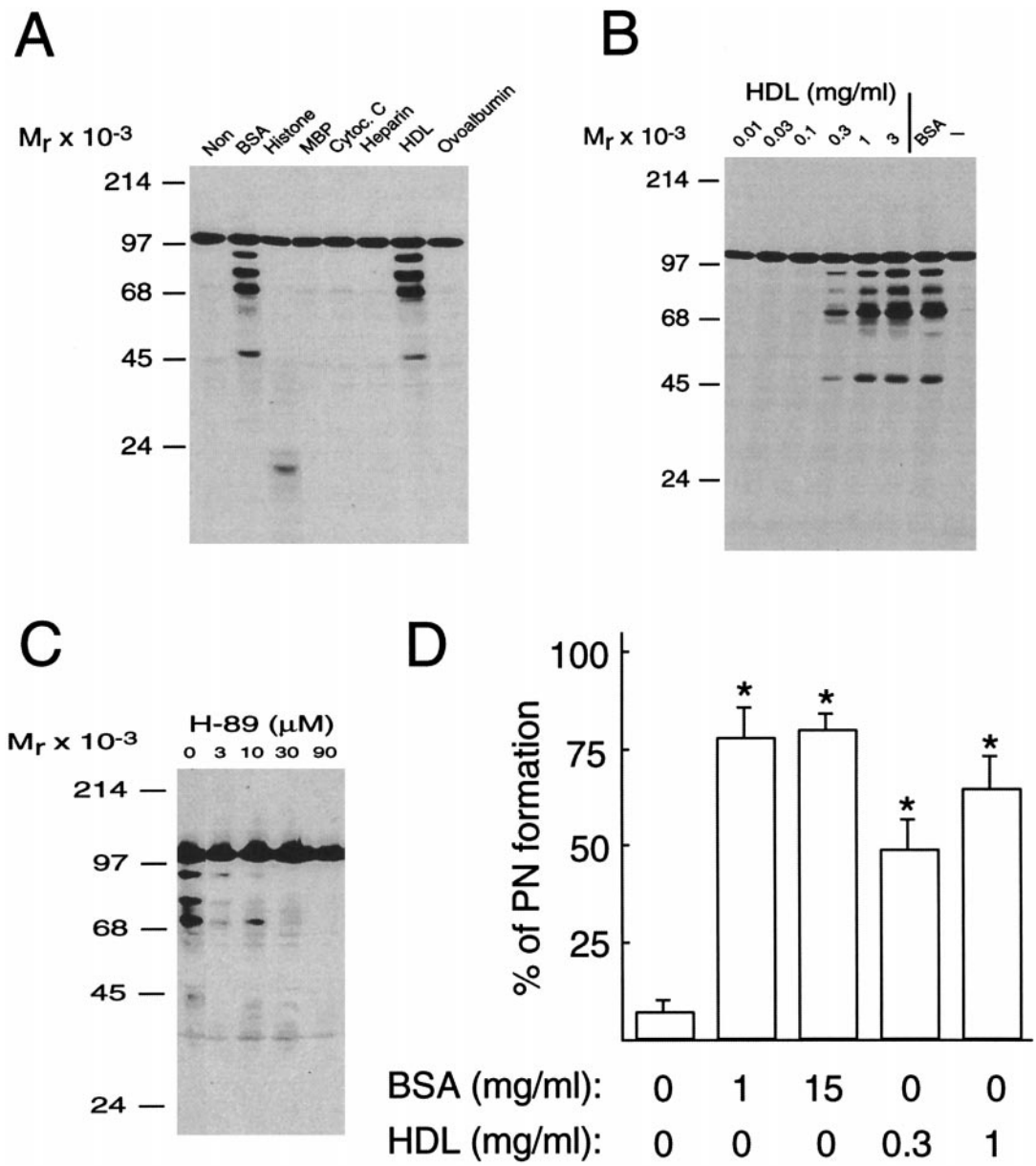

FIG. 6. Effect of HDL, other proteins, and heparin on the capacitation-associated increase in protein tyrosine phosphorylation and capacitation of mouse sperm. (A) Cauda epididymal mouse sperm were collected as described under Materials and Methods and incubated in HMB medium devoid of BSA, but in the presence of 3 mg/ml of the different proteins or heparin listed. Medium containing BSA served as a positive control. After 1.5 h of incubation the pattern of protein tyrosine phosphorylation was analyzed by PAGE and immunoblotted using anti-phosphotyrosine antibodies. (B) Sperm were incubated in HMB medium devoid of BSA and in the absence or presence of different concentrations of HDL as listed. One of the samples was incubated in the presence of 3 mg/ml BSA as a positive control. After 1.5 h of incubation the pattern of protein tyrosine phosphorylation was analyzed by PAGE and immunoblot using anti-phosphotyrosine antibodies. (C) Sperm were incubated in HMB medium containing 3 mg/ml HDL and increasing concentrations of H-89. After 1.5 h of incubation, the pattern of protein tyrosine phosphorylation was analyzed by PAGE and immunoblot using anti-phosphotyrosine antibodies. The experiments were performed at least three times with similar results. Shown are representative experiments. (D) Sperm were capacitated for 2 h in media containing the indicated concentrations of either BSA or HDL and then incubated with metaphase II-arrested eggs for 3 h. Following insemination the eggs were observed for signs of fertilization as described under Materials and Methods. Data represent the means ± SEM of five independent experiments in which a minimum of 30 eggs was used for each experimental condition. $*P < 0.001$ compared to the control without addition of protein.

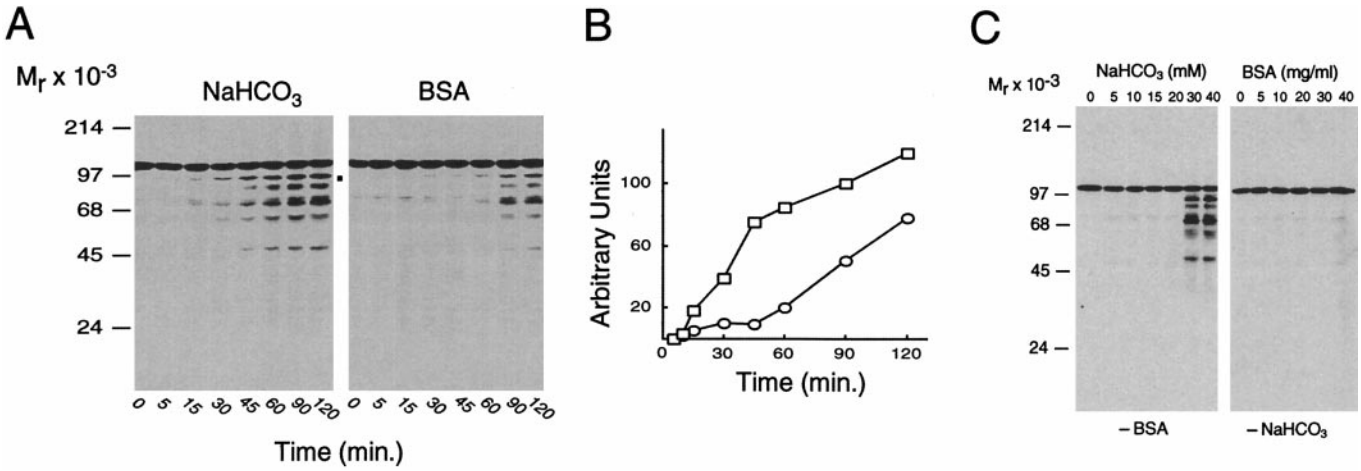

FIG. 7. Recovery of protein tyrosine phosphorylation after incubation of sperm in medium lacking $NaHCO_3$ or BSA. (A) Cauda epididymal mouse sperm were collected as described under Materials and Methods and incubated in HMB medium devoid of BSA (BSA) or devoid of $NaHCO_3$ ($NaHCO_3$) for 1 h. After 1 h of incubation, BSA and $NaHCO_3$ were added to the final concentrations of 3 mg/ml and 10 mM, respectively, to the medium lacking the respective component, and the incubation continued for the times indicated. The sperm were then analyzed by PAGE and immunoblotted using anti-phosphotyrosine antibodies. This experiment was performed at least three times with similar results, and a representative experiment is shown. The small dot between the two blots indicates the location of a M_r 95,000 phosphotyrosine-containing protein. (B) The M_r 95,000 band in A was scanned and quantified using NIH Image software. The data are depicted in arbitrary units. Circles represent BSA supplementation; squares represent $NaHCO_3$ supplementation. (C) Effect of increasing concentrations of $NaHCO_3$ and of BSA on the pattern of protein tyrosine phosphorylation of sperm incubated, respectively, in the absence of BSA or $NaHCO_3$. Cauda epididymal mouse sperm were collected as described under Materials and Methods and incubated in HMB medium devoid of BSA (−BSA) and increasing concentrations of $NaHCO_3$ or incubated in HM medium devoid of $NaHCO_3$ (−$NaHCO_3$) and increasing concentrations of BSA. The sperm were then analyzed by SDS–PAGE and immunoblotted using anti-phosphotyrosine antibodies. This experiment was performed at least three times with similar results, and a representative experiment is shown.

an increase in protein tyrosine phosphorylation of a subset of proteins. Moreover, the up-regulation of protein tyrosine phosphorylation by cAMP and PK-A represents a unique mode of signal transduction crosstalk that, to date, has only been observed in sperm. Since sperm capacitation in many species can be accomplished *in vitro* in defined medium without the addition of biological fluids, it is likely that there is an intrinsic regulatory component of capacitation that involves preprogrammed membrane, transmembrane, and/or intracellular signaling events that, once initiated, lead to the capacitated state. This does not rule out the possibility of higher order regulation of this "intrinsic" process(es) during capacitation *in vivo* by components of the male and female reproductive tracts.

In the present study we further examined the mechanisms by which these tyrosine phosphorylations are regulated, specifically with respect to how the BSA present in the capacitation medium regulates these phosphorylation processes and capacitation. Several authors (Davis, 1976, 1981; Davis *et al.*, 1979; Go and Wolf, 1985; Langlais and Roberts, 1985) have suggested that BSA is necessary in the capacitation medium for the removal of cholesterol from the sperm plasma membrane, and other investigations have shown that cholesterol loss can occur during capacitation (Cross, 1996; Cross and Razy-Faulkner, 1997; Lin and Kan, 1996; Suzuki and Yanagimachi, 1989). Using different approaches, we demonstrate here that capacitation is associated with a loss of cholesterol from the sperm plasma membrane and that BSA, in some way, promotes the loss of this steroid. The loss of this steroid leads to the activation of this aforementioned unique signal transduction cascade by an, as yet, undefined mechanism.

In this report we demonstrated that the presence of cholesterol-SO_4^- blocked the ability of BSA to initiate the capacitation-associated increases in protein tyrosine phosphorylation. This effect was specific for the cholesterol moiety of the cholesterol-SO_4^- molecule, since pregnenolone-SO_4^- and androstenedione-SO_4^- were not able to mimic the cholesterol-SO_4^- effect even at high concentrations. Although cholesterol was able to reduce the BSA-dependent increase in protein tyrosine phosphorylation, it is likely that the limited solubility of cholesterol accounted for its reduced effectiveness compared to the more soluble cholesterol-SO_4^-. The observation that additional BSA can overcome the cholesterol-SO_4^--induced inhibition of protein tyrosine phosphorylation demonstrates that cholesterol-SO_4^- is not irreversibly affecting sperm viability. In addition, cAMP agonists such as IBMX and dibutyryl cAMP are also able to overcome the cholesterol-SO_4^- inhibition of protein tyrosine phosphorylation, demonstrating that the cholesterol-SO_4^- effect is upstream of PK-A activation in this signal transduction cascade.

We also demonstrated that cholesterol-SO_4^- inhibits both the spontaneous as well as the ZP-induced acrosome reaction. This result further supports the correlation between the activation of this unique signal transduction pathway and those processes associated with capacitation. We must emphasize that although these two events are tightly correlated, we have not demonstrated that protein tyrosine phosphorylation is necessary and/or sufficient for capacitation. The tight correlation between these two events has also been demonstrated in sperm from other species such as bull (Galantino-Homer *et al.*, 1997), human (Carrera *et al.*, 1996; Leclerc *et al.*, 1996; Luconi *et al.*, 1996), horse (Rosenberger *et al.*, 1998), pig (Kalab *et al.*, 1998), and hamster (Visconti *et al.*, 1999b). These results suggest that the PK-A induced up-regulation of protein tyrosine phosphorylation associated with capacitation may be universal for mammalian sperm.

These data represent one of the first reports describing the regulation of an intracellular signal transduction pathway by cholesterol. Stulnig *et al.* (1997) have demonstrated that signal transduction via CD59 and CD48 in Jurkat T cells leading to an increase in intracellular calcium is regulated, in some manner, by cellular cholesterol. This effect, however, appears to be independent of effects on membrane dynamics, which is unlike the case that we report here. The aforementioned reduction in sperm membrane cholesterol accompanying capacitation has been demonstrated to result in a decrease in the cholesterol/phospholipid ratio as assessed by a variety of criteria (Hoshi *et al.*, 1990; Tesarik and Flechon, 1986). Such changes likely account for the observed alterations in sperm membrane fluidity (Wolf *et al.*, 1986a), the aggregation of intramembranous particles and formation of particle-free patches (Koehler and Gaddum-Rose, 1975), and the documented membrane protein redistributions reported with lectins (Cross and Overstreet, 1987) and antibodies (Rochwerger and Cuasnicu, 1992; Shalgi *et al.*, 1990) observed during this maturational event. From the standpoint of cell signaling, this change in membrane dynamics may have profound effects on transmembrane signaling and may represent some of the "intrinsic" control of capacitation described above. Transmembrane signaling may be initiated by changes in ion channel activity and/or the activity of membrane-associated enzymatic and nonenzymatic proteins. In addition, this dramatic change in plasma membrane lipid architecture could also be functionally important, as it may ultimately prime the membrane for fusion with the outer acrosomal membrane during the acrosome reaction, an endpoint of capacitation and a prerequisite to successful fertilization. Since cholesterol efflux appears to be the driving force behind these changes in membrane dynamics during capacitation, a clear understanding of the mechanism by which this steroid moves within the plasma membrane and out of the plasma membrane in response to an appropriate acceptor is critical to a molecular understanding of this maturational event.

Our lab has demonstrated that this release of cholesterol is, in some manner, tied to changes in protein tyrosine phosphorylation. The initiation of signal transduction pathways that result in the activation of tyrosine kinases and protein tyrosine phosphorylation normally involves plasma membrane receptors. These receptors could be tyrosine kinases or could be receptors that associate with tyrosine kinases. Sperm represents a unique case in which the increase in protein tyrosine phosphorylation is regulated through a cAMP and PK-A pathway. We have examined this pathway in greater detail in this report and demonstrate that PK-A activity is significantly higher when the sperm are incubated in the presence of BSA. How cholesterol removal regulates such a pathway is not known. One could speculate that the removal of cholesterol from the sperm plasma membrane could alter membrane dynamics and increase the permeability of the sperm to certain ions, such as HCO_3^- and/or Ca^{2+}, which are capable of stimulating the sperm adenylyl cyclase. The sperm adenylyl cyclase has many regulatory properties that set it apart from somatic cell adenylyl cyclases, one notable difference being its ability to be directly activated by HCO_3^- (Garty and Salomon, 1987; Okamura *et al.*, 1985; Visconti *et al.*, 1990, 1995b). We and others have demonstrated that these ions are necessary for both the increase in protein tyrosine phosphorylation and the capacitation process (Neill and Olds-Clarke, 1987; Visconti *et al.*, 1995a), and we have also demonstrated that the effect of these ions is upstream of the cAMP/PK-A pathway (Visconti *et al.*, 1995b, 1997). In the present work we demonstrated that the increase in protein tyrosine phosphorylation recovered slowly after adding back BSA to sperm incubated for 1 h in medium devoid of this protein and recovered almost immediately after adding back HCO_3^- to sperm incubated for 1 h in medium devoid of this anion. These observations could be explained by a model in which cholesterol removal by BSA would prime the membrane for an increased permeability to HCO_3^-. Consistent with this model is the fact that when sperm are incubated in the presence of high concentrations of $NaHCO_3$, we observed a BSA-independent increase in protein tyrosine phosphorylation, suggesting again that the HCO_3^- site of action is downstream of the BSA effect. This model, if correct, could open new questions regarding the role of cholesterol in the regulation of ion movements. In this respect it is interesting to note that capacitation has also been correlated with the hyperpolarization of the plasma membrane (Arnoult *et al.*, 1999; Zeng *et al.*, 1995). It is not yet known if this hyperpolarization is dependent or independent of cholesterol removal by BSA. Since plasma membrane hyperpolarization could be either upstream or downstream of the increased permeability to HCO_3^-, the possibility that anion channels could be regulated by membrane potential is an important consideration for future research.

Yet another question pertains to the mechanisms regulating sperm cholesterol efflux. In our *in vitro* system, BSA is functioning as an extracellular acceptor for sperm plasma membrane cholesterol. Based on the results of several

investigators studying cholesterol efflux in sperm during capacitation, one could postulate that such an efflux mechanism might bear some similarities to reverse cholesterol transport observed in somatic cells, where the first step is the efflux of cellular cholesterol to an appropriate extracellular acceptor, usually HDL. It is generally accepted that cholesterol efflux occurs by an aqueous diffusion mechanism in which the cholesterol molecules de-adsorb from the plasma membrane into the aqueous phase, diffuse, and are then solubilized by an acceptor molecule. Since HDL can induce the increase in protein tyrosine phosphorylation through a cAMP/PK-A pathway in sperm (this report) and can support capacitation (this report; Lane *et al.*, 1999; Therien *et al.*, 1997), it is likely that BSA and HDL may function similarly as cholesterol acceptors in sperm leading to capacitation *in vitro.*

Recently, our laboratory has demonstrated that the cholesterol-binding heptasaccharides, methyl-β-cyclodextrin and OH-propyl-β-cyclodextrin, promote the release of cholesterol from the mouse sperm plasma membrane in media devoid of BSA, promote protein tyrosine phosphorylation, and support capacitation and *in vitro* fertilization (Visconti *et al.*, 1999a). Both of these β-cyclodextrins were also demonstrated to increase protein tyrosine phosphorylation in the absence of BSA in bovine (Visconti *et al.*, 1999a) and human (Osheroff *et al.*, 1999) sperm. Independently, Choi and Toyoda (1998) and Cross (1999) demonstrated that β-cyclodextrins were able to capacitate mouse and human sperm, respectively, in the absence of BSA. These data provide additional support for the existence, function, and physiological role of cholesterol release in sperm cell signaling.

If one accepts the notion that capacitation *in vivo* is also associated with cholesterol loss, it is important to consider what component of the female reproductive tract might serve as a cholesterol acceptor *in vivo.* It is clear that the composition of the fluids of the female tract arise, in part, as a transudate of the serum so that serum-derived sterol acceptors/binding proteins could function *in vivo.* The identity of such acceptors remains to be clarified.

In conclusion, this work demonstrates that cholesterol efflux from the sperm plasma membrane can initiate a signal transduction pathway that leads to an increase in protein tyrosine phosphorylation through the activation of PK-A. Our efforts will be directed toward the identification of the molecular mechanisms involved in the regulation of this unique signal transduction pathway as well as to the characterization of the role of protein tyrosine phosphorylation in regulating sperm function.

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