Cholesterol Efflux-mediated Signal Transduction in Mammalian Sperm

β-CYCLODEXTRINS INITIATE TRANSMEMBRANE SIGNALING LEADING TO AN INCREASE IN PROTEIN TYROSINE PHOSPHORYLATION AND CAPACITATION

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Sperm capacitation in vitro is highly correlated with an increase in protein tyrosine phosphorylation that is regulated by cAMP through a unique mode of signal transduction cross-talk. The activation of this signaling pathway, as well as capacitation, requires bovine serum albumin (BSA) in the incubation medium. BSA is hypothesized to modulate capacitation through its ability to remove cholesterol from the sperm plasma membrane. Here we demonstrate 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. Both of these β-cyclodextrins were also demonstrated to increase protein tyrosine phosphorylation in the absence of BSA in both mouse and bull sperm, and the patterns of phosphorylation were similar to those induced by media containing BSA. The potency of the different β-cyclodextrins to increase protein tyrosine phosphorylation in sperm was correlated with their cholesterol binding efficiencies, and preincubation of the β-cyclodextrins with cholesterol-SO\textsuperscript{−} to saturate their cholesterol-binding sites blocked the ability of these compounds to stimulate protein tyrosine phosphorylation. The β-cyclodextrin effect on protein tyrosine phosphorylation was both NaHCO\textsubscript{3} and protein kinase A-dependent. The β-cyclodextrins were also able to capacitate mouse sperm in the absence of BSA, as measured by the ability of the zona pellucida to induce the acrosome reaction and by successful fertilization in vitro. In summary, β-cyclodextrins can completely replace BSA in media to support signal transduction leading to capacitation. These data further support the coupling of cholesterol efflux to the activation of membrane and transmembrane signaling events leading to the activation of a unique signaling pathway involving the cross-talk between cAMP and tyrosine kinase second messenger systems, thus defining a new mode of cellular signal transduction initiated by cholesterol release.

Immediately after ejaculation, mammalian sperm do not possess the ability to fertilize the egg. They acquire the ability to fertilize during transit through the female tract in a poorly understood process known as capacitation (1–4). Capacitation is defined as the time-dependent acquisition of fertilization competence, and is generally assessed as the ability of the acrosome-intact sperm to undergo an acrosome reaction in response to physiological inducers such as the ZP\textsuperscript{1} or progesterone (5, 6), or by the ability of the sperm to fertilize eggs (7).

Capacitation can be mimicked in vitro by incubating epididymal or ejaculated sperm in a defined medium containing appropriate concentrations of electrolytes, metabolic energy sources, Ca\textsuperscript{2+}, HCO\textsubscript{3}\textsuperscript{−}, and a protein source (usually bovine serum albumin; BSA) (7). Capacitation has also been correlated with changes in sperm intracellular ion concentrations, metabolism, and motility (7, 8). Although these changes have been known for many years to accompany this maturational event, the molecular basis underlying these events is poorly understood. Remarkably, capacitation in vitro can occur in the absence of any specific external stimulus, suggesting that specific aspects of the capacitation process can be initiated and controlled intrinsically by the sperm itself, and that certain minimal environmental requirements must be met. This intrinsic nature of the capacitation process is of great interest from a cell regulation standpoint. It is possible that a controlling factor(s) intrinsic to the sperm plasma membrane may regulate changes in the properties of the membrane and that a de-repression of a set of preprogrammed cellular events must ultimately occur to promote the development of the capacitated state. The requirement for BSA in regulating capacitation is thought to be due to its ability to remove cholesterol from the sperm plasma membrane (9–14). It is proposed that cholesterol efflux then

1 The abbreviations used are: ZP, zona pellucida; BSA, bovine serum albumin; PKA, protein kinase A; M-β-CD, methyl-β-cyclodextrin; 2-OH-p-β-CD, OH-propyl-β-cyclodextrin; PVA, polyvinyl alcohol; PAGE, polyacrylamide gel electrophoresis; IBMX, isobutylmethylxanthine; Bt\textsubscript{2}cAMP, dibutyryl cAMP.
leads to changes in membrane architecture and fluidity that give rise to the capacitated state. Cholesterol efflux, therefore, may represent an integral part of this intrinsic regulatory property of sperm to undergo capacitation. Previous studies have demonstrated that sperm capacitation in several species is correlated with an increase in tyrosine phosphorylation of a subset of proteins (15–18). Our laboratory has determined that both protein tyrosine phosphorylation and capacitation are regulated by cAMP at the level of protein kinase A (PKA), and requires the presence of Ca$^{2+}$, HCO$_3^-$, and BSA in the medium (19). This mode of signal transduction cross-talk is, to date, unique to sperm. Although BSA is believed to be required for capacitation as a consequence of its ability to serve as a cholesterol-binding molecule, it is still not known if the only action of BSA is through the removal of cholesterol.

**β-Cyclodextrins are cyclic heptasaccharides consisting of β(1-4)-glucopyranose units (20). These compounds are water-soluble, are able to effectively solubilize non-polar substances, and because of these properties, have been used to deliver hydrophobic drugs (20). These compounds are also able to promote cholesterol efflux from a variety of somatic cells (21, 22). Yancey et al. (21) demonstrated that the order of potency in accepting cholesterol is methyl-β-cyclodextrin (M-β-CD) > OH-propyl-β-cyclodextrin (2-OH-p-β-CD) > β-cyclodextrin. These results also showed that there is an initial rapid efflux of cholesterol from the plasma membrane of mouse L-cells and human fibroblasts in response to β-cyclodextrins (21).

In the present study, we tested the hypothesis that BSA is acting through the removal of cholesterol from the sperm plasma membrane by analyzing if β-cyclodextrins are able to replace BSA in the medium to up-regulate signal transduction pathways leading to protein tyrosine phosphorylation as well as capacitation. We demonstrate that both M-β-CD and 2-OH-p-β-CD promote the release of cholesterol from the mouse sperm plasma membrane in the absence of BSA. Addition of these β-cyclodextrins to the medium also increased protein tyrosine phosphorylation in the absence of BSA in mouse and bull sperm, and the patterns of phosphorylation were similar to those patterns seen in media containing BSA. These results indicate that β-cyclodextrins are likely due to their ability to capture cholesterol from the sperm plasma membrane. The β-cyclodextrin effect on protein tyrosine phosphorylation was dependent on the presence of NaHCO$_3$ in the capacitation medium and the effect was cAMP-dependent. β-Cyclodextrins were also able to capacitate mouse sperm in the absence of BSA, as measured by the ability of the ZP to induce the acrosome reaction and by successful fertilization in vitro. This work is the first to demonstrate that a chemically defined non-protein constituent can substitute in capacitation media for protein cholesterol acceptors. These data further support the idea that cholesterol efflux is, in some way, coupled to the activation of membrane and transmembrane signaling events leading to the activation of a unique cross-talk between cAMP and tyrosine kinase second messenger systems, and thus defines a new mode of cellular signal transduction.

**EXPERIMENTAL PROCEDURES**

**Materials**—2-OH-propyl-β-cyclodextrin, methyl-β-cyclodextrin, and BSA (Fraction V, Sigma) were purchased from Sigma. Anti-phosphotyrosine antibody (clone 4G10) was from UBI, Lake Placid, NY. Solvents were from EM Science (chromatographic grade). Analytical diphase plates (Unibond RP$_2$/silica gel, 10 × 12 cm, 250 μm thickness) were obtained from Analtech Inc. (Newark, DE). Whatman HP-K silica gel plates (10 × 10 cm, 250 μm thickness) were purchased from Whatman Inc. (Clifton, NJ).

**Culture Media**—The basic medium used for the studies with mouse sperm was a modified Krebs-Ringer bicarbonate medium (HMB-Hepes buffered), as described by Lee and Storey (23). 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 gentle aeration with 5% CO$_2$ in air for 30 min.

A modified Tyrode’s Heps-buffered medium (SpTALPH) was used for the experiments with bull sperm, as described by Parrish et al. (24). This medium was modified in the following manner: BSA was replaced by 1 mg/ml polyvinyl alcohol (PVA) to reduce the ability of SpTALPH to capacitate sperm (15) and 0.4 mM EDTA was added. The medium was further modified in the absence of Ca$^{2+}$ and pyruvate and frozen at −20 °C in aliquots for single use. Working complete SpTALPH medium was prepared by adding Ca$^{2+}$ (2 mM), pyruvate (1 mM), NaHCO$_3$ (10 mM), PVA (1 mg/ml), and gentamycin (50 mg/ml). The pH of SpTALPH was adjusted to 7.4 following equilibration for 1 h at 39 °C in room air, and sterilized by passage through a 0.20-μm filter (Nalgene, Fisher, Pittsburgh, PA).

**Preparation of Mouse Sperm**—Caudal epididymal mouse sperm were collected from CD1 retired breeder males by placing one mated cauda epididymis in 0.5 ml of medium HMB without BSA at 30 °C. After 5 min the sperm in suspension were washed in 10 ml of the same medium by centrifugation at 800 × g for 10 min at room temperature (24 °C). The sperm were then resuspended to a final concentration of 5–10 × 10$^6$ 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 centrifuged at 5,000 × g for 1 min (room temperature), the sperm pellet washed in 1 ml of phosphate-buffered saline, centrifuged again, and the resultant pellet resuspended in sample buffer (25) without mercaptoethanol and boiled for 5 min. After centrifugation at 5,000 × g for 5 min, the supernatant was removed, 2-mercaptoethanol was added to a final concentration of 5% (v/v), the samples boiled for 5 min, and then subjected to SDS-PAGE as described below.

**Preparation of Bovine Sperm**—Ejaculated bovine sperm (Bos taurus, Holstein), collected by artificial vagina, was generously provided by the Hofmann Center at New Bolton Center, University of Pennsylvania School of Veterinary Medicine (Kennett Square, PA). The sperm were immediately assessed for motility by light microscopy and then, at 10–20 min following collection, were diluted 1:5 into SpTALPH. These samples were then subjected to two washes by centrifugation at 375 × g for 10 min each in SpTALPH to remove seminal plasma, as described previously (17, 24). Sperm were kept at 22–24 °C in SpTALPH following the washes for transport to the laboratory (1–2 h), and then subjected to one wash in SpTALPH. The concentration of sperm in the SpTALPH solution was determined by hemocytometer and adjusted to 5 × 10$^6$ cells/ml with SpTALPH before initiating the capacitation experiments. For capacitation, 100-μl aliquots of the sperm suspension (5 × 10$^6$ cells), as well as the appropriate test reagents (1–3 μl), were incubated in capped 1.5-ml polypropylene microcentrifuge tubes at 39 °C in a Thermolyne 37900 culture as described previously (17, 24). Additions to the sperm suspension were made immediately before the start of the incubation period. Following an incubation period of 4 h, the sperm were centrifuged at 10,000 × g for 3 min at room temperature, washed in 1 ml of phosphate-buffered saline containing 0.2 mM Na$_2$VO$_4$ at room temperature, and the sperm pellet then resuspended in sample buffer (25) without mercaptoethanol and boiled for 5 min. After centrifugation at 5,000 × g for 5 min, the supernatant was removed, 2-mercaptoethanol was added to a final concentration of 5% (v/v), the samples boiled for 5 min, and then subjected to SDS-PAGE as described below. For all experiments, sperm from two to three different mature Holstein bulls were assayed to control for individual variation.

**Steroid Measurements**—Mouse sperm (5 × 10$^6$) were incubated in 500 μl of capacitation medium in either the absence or presence of 3 mg/ml BSA or with the appropriate β-cyclodextrins for 1.5 h. After this period, each aliquot was centrifuged for 10 min at 10,000 × g and cholesterol, desmosterol, and cholesterol-SO$_4$ were measured in the sperm pellet and in the resultant medium supernatant as described previously (26). Briefly, sperm pellets were extracted with 20 volumes of chloroform/methanol (1:1, v/v) for 5 s, vortexed for 10 s, centrifuged at 800 × g for 3 min, and the supernatant evaporated to dryness under N$_2$. The remainder of the supernatants were made immediately before use by the addition of 6 volumes of chloroform/methanol (2:1, v/v) for 3 min, vortexed for 10 s, centrifuged at 800 × g for 3 min, and the lower organic phase aspirated and evaporated to dryness. Both the sperm pellet and medium supernatant extracts were dissolved in 20 μl of chloroform/methanol (1:1, v/v), and 4-μl aliquots applied to silver nitrate-impregnated Whatman HP-K silica gel microplates (Whatman Inc., Clifton, NJ) (5 × 10$^6$).
5 cm, 250 μm thickness). Aliquots (4 μl) of cholesterol, desmosterol, cholesterol sulfate, and desmosterol (Sigma) at a concentration of 0.1 mg/ml, were applied on separate lanes as reference standards. The plates were pre-developed in chloroform/methanol (1:1, v/v) to 1 cm from the lower edge of the plate. This pre-development step was minimizes eddy diffusion which results in band broadening and lower resolution. Following pre-development, 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 Flute 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 cholesterol, desmosterol, and cholesterol sulfate standards, and the values expressed as nanograms/10^6 cells.

**SDS-PAGE and Immunoblotting**—SDS-PAGE (25) was performed in 8 or 10% gels. Electrophoretic transfer of proteins to Immobilon P (Millipore) in all experiments was carried out according to the method of Towbin et al. (27) at 70 V (constant) for 2 h at 4 °C. Immunodetection was carried out at room temperature as described previously (28) using a monoclonal antibody against anti-phosphotyrosine (clone 4G10; UbI) and blots developed using enhanced chemiluminescence detection with an ECL kit (Amersham International, according to manufacturer's instructions).

**Coomassie Blue Assay for the Acrosome Reaction**—As one end point of capacitation, we analyzed the ZP-induced acrosome reaction in mouse sperm, based on the premise that only capacitated sperm would undergo exocytosis in response to ZP. The percentage of acrosome reactions was measured using Coomassie Blue G-250 as described by Thaler and Cardullo (29). Briefly, sperm were incubated under the desired experimental conditions for 1.5 h, followed by the addition of 5 ZP/ml, 5 μl A23187, or buffer for an additional 30 min. An equal volume of 2 × fixative solution (7.5% formaldehyde in phosphate-buffered saline) 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 mM ammonium acetate (pH 9). After centrifugation, the sperm pellet was resuspended in 20–50 μl of transfection buffer, spread on to poly-L-lysine-coated slides, and air dried. The slides were then stained with 0.04% (w/v) Coomassie Blue G-250 in 3.5% (v/v) perchloric acid for 10 min, gently rinsed with deionized H₂O until they appeared blue, air dried, and then 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 different experiments.

**In Vitro Fertilization**—*In vitro* fertilization of metaphase II-arrested mouse eggs was performed as described previously (30), with modifications as described in Visconti et al. (15). PVA was used to maintain the isotonic conditions when sperm were cultured in media devoid of BSA prior to insemination. When β-cyclodextrins were included in the capacitation media, the medium containing the capacitated sperm were diluted 100-fold prior to insemination of the eggs. β-Cyclodextrins were also present in the insemination media in some of the experiments. All experiments were done by directly measuring the cholesterol released into the incubation medium, as well as that remaining associated with the sperm after incubating the sperm in the absence or presence of these compounds. Cauda epididymal sperm were incubated in HMB medium in the absence or presence of 3 mg/ml BSA, 1 or 3 mM 2-OH-p-β-CD, and 1 or 3 mM M-β-CD. This is in contrast to sperm incubated in media containing 3 mg/ml BSA for the same period of time, where a significant release of these steroids into the media was observed. 2-OH-p-β-CD, which is a very effective cholesterol binding heptasaccharide, mediated steroid release from sperm in a concentration-dependent manner in the absence of BSA (Table I). This β-cyclodextrin was more effective than BSA in mediating steroid release from the sperm, as evidenced by the significantly higher steroid concentrations observed in the media following incubation. M-β-CD, likewise, mediated steroid release from sperm in a concentration-dependent manner, but was more potent than 2-OH-p-β-CD, consistent with the fact that M-β-CD is the most potent β-cyclodextrin with respect to its affinity for cholesterol binding (21).

**Effects of 2-OH-propyl-β-cyclodextrin and Methyl-β-cyclodextrin on Sperm Protein Tyrosine Phosphorylation in Media Devoid of BSA**—As mentioned above, BSA is proposed to act through its ability to serve as an acceptor for cholesterol and by promoting cholesterol efflux from the sperm plasma membrane. Since the presence of BSA in the capacitation medium was required for the capacitation-associated increase in protein tyrosine phosphorylation, we hypothesized that BSA could work through its ability to sequester cholesterol. Therefore, we examined whether cholesterol removal by β-cyclodextrins in medium devoid of BSA was sufficient to activate the signal transduction pathways that lead to an increase in protein tyrosine phosphorylation. Cauda epididymal sperm were incubated for 1.5 h in BSA-free medium in the absence or presence of various concentrations of 2-OH-p-β-CD and M-β-CD. As shown in Fig. 1, both β-cyclodextrins were able to

| Treatment | Fraction | Steroids |
|-----------|----------|----------|
| Sperm     | C        | D        | CSO₄⁻|
| 437 ± 4   | 253 ± 5  | 15 ± 1   |        |
| Media     | 11 ± 1   | 5 ± 1    | ND     |
| BSA (3 mg/ml) | 355 ± 3   | 197 ± 7  | 9 ± 1  |
| Sperm     | 98 ± 3   | 57 ± 5   | 6 ± 1  |
| 2-OH-p-β-CD | 179 ± 3   | 94 ± 3   | 1 ± 0.2|
| (3 mM)    | 287 ± 8  | 157 ± 13 | 9 ± 1  |
| 2-OH-p-β-CD | 120 ± 3   | 60 ± 2   | 7 ± 1  |
| (3 mM)    | 305 ± 9  | 173 ± 2  | 8 ± 1  |
| M-β-CD    | 168 ± 7  | 114 ± 3  | 7 ± 0.5|
| (1 mM)    | 280 ± 4  | 137 ± 2  | 8 ± 1  |
| M-β-CD    | 32 ± 0.6 | 15 ± 0.3 | 3 ± 0.3|
| (3 mM)    | 410 ± 8  | 239 ± 5  | 13 ± 1 |
Effects of Cholesterol Sulfate Incubation with β-Cyclodextrins on Their Ability to Stimulate Protein Tyrosine Phosphorylation—As stated earlier, β-cyclodextrins such as M-β-CD and 2-OH-p-β-CD both possess the ability to serve as cholesterol acceptors. If the β-cyclodextrins effects observed on protein tyrosine phosphorylation were coupled to their ability to sequester cholesterol, it would be predicted that these compounds would lose their effects on protein tyrosine phosphorylation if their steroid binding capacity was abrogated by preincubation with cholesterol-SO₄₂⁻. As shown in Fig. 3, preincubation of either M-β-CD or 2-OH-p-β-CD with increasing concentrations of cholesterol-SO₄₂⁻ inhibited the ability of the β-cyclodextrins to support sperm protein tyrosine phosphorylation. A similar inhibitory effect on protein tyrosine phosphorylation was observed following preincubation of 2-OH-p-β-CD with cholesterol (Fig. 3). Taken together, these data suggest that the β-cyclodextrins are inducing an increase in sperm protein tyrosine phosphorylation by their ability to function as cholesterol acceptors.
Hatched bars, those observed with 2-OH-p-cyclodextrin in medium devoid of BSA (Fig. 4). Incubation of sperm in BSA-free medium containing various concentrations of 2-OH-propyl-cyclodextrin resulted in an increase in percentages of both spontaneous and ZP-induced acrosome reactions (Fig. 4). This increase in the percentage of spontaneous acrosome reactions increased dramatically at concentrations of 2-OH-p-β-CD above 1 mM (Fig. 5), suggesting that higher concentrations of this β-cyclodextrin may render these sperm extremely labile so that they undergo exocytosis spontaneously; this would also be consistent with the toxic effects of these compounds at higher concentrations. Likewise, M-β-CD displayed a concentration-dependent ability to increase the percentages of spontaneous acrosome reactions (data not shown). These effects were more potent than those observed with 2-OH-p-β-CD, consistent with the difference in potency of these two β-cyclodextrins to bind cholesterol.

Although the ZP-induced acrosome reaction is one end point of capacitation, fertilization still represents the most definitive end point of the maturational process (5, 7). We examined whether sperm incubated in media devoid of BSA but containing 2-OH-p-β-CD supported capacitation with respect to their ability to fertilize eggs in vitro. The success of in vitro fertilization was monitored by the formation of both the male and female pronuclei. As previously demonstrated, sperm incubated in the absence of BSA were unable to fertilize eggs (Fig. 6A). When PVA was substituted for BSA in the capacitation and fertilization media to inhibit the formation of sperm aggregates and prevent sticking of the eggs to various surfaces, PVA-containing media was not demonstrated to increase sperm protein tyrosine phosphorylation (data not shown) or support in vitro fertilization (Fig. 6A). In contrast, sperm incubated in media containing 0.5 and 1 mM 2-OH-p-β-CD were able to fertilize eggs (Fig. 6A), demonstrating that this β-cyclodextrin can support capacitation of the sperm in the absence of BSA. In contrast, in vitro fertilization performed with sperm incubated in medium containing higher concentrations of 2-OH-p-β-CD (3 mM) displayed reduced fertilization rates (Fig. 6B), likely due to the increased incidence of spontaneous acrosome reactions (see Fig. 5); acrosome reacted sperm will not bind to the ZP and, therefore, are unable to fertilize ZP-intact eggs.

Role of Bicarbonate and Protein Kinase A in Mediating the β-Cyclodextrin Effect on Mouse Sperm Protein Tyrosine Phosphorylation—Work from our laboratory (17, 19, 32) and others (18) has demonstrated that activation of PKA is an important...
component of the capacitation process. We have also demonstrated that the elevation of cAMP and activation of PKA up-regulates protein tyrosine phosphorylation during capacitation (17, 19). It is hypothesized that the BSA-mediated removal of cholesterol from the sperm plasma membrane alters membrane dynamics such that changes in HCO\textsubscript{3} and Ca\textsuperscript{2+} conductance occur, leading to an activation of the sperm adenyl cyclase, an elevation of cAMP, activation of PKA, and an increase in protein tyrosine phosphorylation (19, 32). Since β-cyclodextrins can support protein tyrosine phosphorylation and capacitation, we examined whether the effects of these compounds on protein tyrosine phosphorylation were regulated in some manner by HCO\textsubscript{3} and PKA. In contrast to sperm incubated in HCO\textsubscript{3}-containing medium plus 3 mM 2-OH-p-β-CD, incubation of sperm in HCO\textsubscript{3}-free medium containing 3 mM 2-OH-p-β-CD did not display protein tyrosine phosphorylation (Fig. 7). Moreover H-89, an inhibitor of PKA, inhibited the protein tyrosine phosphorylation induced by 3 mM 2-OH-p-β-CD in medium containing HCO\textsubscript{3} in a concentration-dependent manner with an IC\textsubscript{50} of −1 μM (Fig. 7), consistent with the concentration needed to block cellular activation of the PKA pathway leading to protein tyrosine phosphorylation in sperm (19). Taken together, these data suggest that the effects of 2-OH-p-β-CD on protein tyrosine phosphorylation require HCO\textsubscript{3} and are mediated through PKA, similar to that seen with BSA (19).

Effects of 2-OH-propyl-β-cyclodextrin and Methyl-β-cyclodextrin on Protein Tyrosine Phosphorylation in Bovine Sperm—A cAMP-dependent increase in protein tyrosine phosphorylation has been demonstrated to accompany sperm capacitation in other species, including the human (18) and bull (17). We also examined whether cyclodextrins could modulate protein tyrosine phosphorylation in bull sperm. Incubation of ejaculated bull sperm in medium devoid of heparin, which would not support protein tyrosine phosphorylations or promote capacitation (17), and in the presence of various concentrations of 2-OH-p-β-CD (Fig. 8A) or M-β-CD (Fig. 8E) had no effects or moderate effects at higher concentrations, respectively, on protein tyrosine phosphorylation. In contrast, when similar experiments were performed in media containing heparin (Fig. 8, B and F), both β-cyclodextrins displayed concentration-dependent increases in protein tyrosine phosphorylation, with M-β-CD being the greatly more effective compound on a molar basis. This difference in potency of the two β-cyclodextrins is consistent with their relative potencies in binding cholesterol. Likewise, the addition of Bt\textsubscript{2cAMP} plus IBMX to media devoid of heparin (Fig. 8, C and G) also supported β-cyclodextrin-induced protein tyrosine phosphorylation. It is interesting to note that the concentration response curves with both β-cyclodextrins were shifted to the left when compared with experiments performed in heparin-containing media alone (compare Fig. 8, C with B, and G with F). Finally, the addition of both β-cyclodextrins to bovine sperm incubated in media containing both heparin and Bt\textsubscript{2cAMP} plus IBMX (Fig. 8, D and H) resulted in an even more potent β-cyclodextrin effect on protein tyrosine phosphorylation than in media containing heparin alone or in media containing Bt\textsubscript{2cAMP} plus IBMX. These data suggest that β-cyclodextrins can replace BSA to increase protein tyrosine phosphorylation in bull sperm. These experiments suggest that, similar to the mouse, the presence of proteins or non-protein compounds that possess the ability to bind bovine sperm membrane cholesterol are essential to initiate signal transduction leading to an increase in protein tyrosine phosphorylation.

DISCUSSION

Although capacitation was initially described approximately 50 years ago, the molecular basis of this extratessicular maturational event is still poorly understood. Nevertheless, recent work by several laboratories is starting to lead to a unified hypothesis of how this event is controlled. Capacitation can occur spontaneously in vitro in defined media without the addition of biological fluids, suggesting that this process can be controlled by the sperm itself. It is clear that some components of the medium play essential roles in the regulation of capacitation. Although serum albumin (usually BSA) is believed to function as a sink for the removal of cholesterol from the sperm plasma membrane (9–14), it is not known whether cholesterol removal represents the only function of BSA in the capacitation process.

In attempts to understand the signaling pathways that regulate capacitation, our laboratory has recently demonstrated that capacitation of mouse, human, and bovine sperm is associated with an increase in protein tyrosine phosphorylation (15–17), and other laboratories have corroborated these results (18, 33–35). In the present work, we further studied the mechanisms by which these capacitation-associated changes in protein tyrosine phosphorylation are regulated, with specific attention being given to the role of cholesterol efflux in regulating this process. Previously, we demonstrated that protein tyrosine phosphorylation and capacitation were both dependent on the presence of BSA in the incubation medium (15). Our working hypothesis is that BSA, by acting as a sink for cholesterol efflux from the sperm plasma membrane, induces an increase in the permeability of sperm to HCO\textsubscript{3} and Ca\textsuperscript{2+} ions. These ions are able to stimulate the activity of the sperm adenyl cyclase, and in this way increase cAMP concentrations and PKA activity leading to an up-regulation of protein tyrosine phosphorylation. We are currently examining several aspects of this hypothesis. For example, the inter-relationship between BSA and cholesterol movement appears to be important in the regulation of protein tyrosine phosphorylation and capacitation, since preloading BSA with cholesterol sulfate to block the ability of BSA to sequester sperm plasma membrane cholesterol inhibits protein tyrosine phosphorylation and capacitation.2

In our previous work, we demonstrated that the capacitation process and an increase in protein tyrosine phosphorylation

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2 P. E. Visconti and G. S. Kopf, unpublished data.
were tightly correlated. The goal of the experiments in this paper was to analyze how the BSA present in the capacitation medium might regulate the increase in protein tyrosine phosphorylation and the capacitation process. Several authors have suggested that BSA is necessary in the capacitation medium for removal of cholesterol from the plasma membrane (9–14). Our results demonstrate that substitution of the BSA in the media with β-cyclodextrins, heptasaccharides with high affinities for cholesterol, dramatically reduced the sperm membrane-associated cholesterol, induced protein tyrosine phosphorylation, and resulted in sperm capacitation.

This work is the first to describe the regulation of a transmembrane signal transduction pathway by cholesterol removal. Normally, the initiation of signal transduction pathways that result in the activation of tyrosine kinases involves plasma membrane receptors; such receptors could be tyrosine kinases or could be associated with tyrosine kinases. Since sperm present a unique case in which the increase in protein tyrosine phosphorylation is regulated through a cAMP pathway, it is possible that the removal of cholesterol would be necessary for those events leading to the synthesis of cAMP. How cholesterol removal regulates this pathway is still not known. It is clear from numerous studies that cholesterol alters the bulk biophysical properties of biological membranes. For example, this steroid can increase the orientation order of the membrane lipid hydrocarbon chains and, as a consequence, can reduce the ability of membrane proteins to undergo conformational changes that may control their functions, due to the fact that the membrane is less fluid. Therefore, high concentrations of cholesterol in the membrane might inhibit membrane protein function. This “indirect” effect of cholesterol on membrane protein function might stabilize those membrane and transmembrane events that are part of the “intrinsic” regulatory nature of capacitation. Cholesterol has also been demonstrated to have “direct” effects by binding to and regulating membrane protein function; such binding may serve to exert a positive or negative modulatory effect on the protein in question. In fact, studies of several membrane-associated ion transporters (e.g., Na⁺,K⁺-ATPase and GABA transporter) by cholesterol support the idea that both direct and indirect effects of this steroid on the regulation of enzyme/ion channel activity could be invoked (36, 37). In the case of capacitation, it is possible that the removal of cholesterol from the sperm plasma membrane could increase the permeability of the sperm to certain ions, such as HCO₃⁻ and/or Ca²⁺, which are capable of stimulating the sperm adenyl cyclase. This remains to be tested experimentally.

In capacitation in vitro, BSA is acting as an extracellular acceptor for cholesterol. The efflux of cholesterol to extracellular...
lar acceptors in somatic cells is normally mediated by high density lipoproteins or specific subpopulations of high density lipoproteins (38–40). 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 solubilized by an acceptor particle (39, 41). In this work we demonstrated that β-cyclohextrins induce the increase in protein tyrosine phosphorylation through a cyclic AMP/cAMP-dependent protein kinase pathway and support capacitation. Moreover, the effects of different β-cyclohextrins to initiate signaling and bring about functional capacitation are directly related to their affinities for capturing cholesterol. Aside from the fact that these data highlight a new and important role for cholesterol in initiating transmembrane signal transduction, these data also suggest that completely defined media devoid of protein could be utilized in the various assisted reproductive technologies to obtain successful fertilization in vitro. It is also interesting to note that these β-cyclohextrins (specifically M-β-CD) are very effective in inducing spontaneous acrosome reactions, as well as reducing sperm motility, thus defining a possible use for these compounds as contraceptive agents.

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