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Cholesterol Efflux Promotes Acrosome Reaction in Goat Spermatozoa[1]

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ABSTRACT

Cholesterol efflux and membrane destabilization play an important role in sperm capacitation and membrane fusion in the acrosome reaction (AR). In this study we establish the effect of cholesterol removal from spermatozoa on acrosomal responsiveness. Mature goat spermatozoa were incubated in BSA-free medium in the presence of β-cyclodextrin (βCD) as cholesterol acceptor. After incubation with 8 mM βCD, 50–60% of cholesterol was released from sperm membranes with no loss in the phospholipid content, and 35% of AR was induced. However, when 30% of cholesterol was lost, this moderate cholesterol decrease was unable to initiate AR. Cholesterol desorption was very rapid, following an exponential kinetics with a half-time of around 10 min, which is in contrast with the slow sigmoidal kinetics of acrosomal responsiveness: around 2 h was required for maximal AR. Our results suggest that cholesterol efflux has a direct influence on the onset of the AR, that is, merely removing cholesterol would trigger the AR.

INTRODUCTION

Mammalian ejaculated spermatozoa are unable to fertilize an egg until they undergo capacitation during their residence in the female reproductive tract or in a suitable medium in vitro. During capacitation, spermatozoa are hyperactivated, and their acrosome becomes destabilized in preparation for the acrosome reaction (AR). Incubating spermatozoa in the continued presence of seminal plasma prevents capacitation and AR. In contrast, removal of seminal plasma makes spermatozoa susceptible to factors that would trigger the AR. Plasma membrane from spermatozoa exposed to seminal plasma, both in vivo and in vitro, contains a series of acidic 15- to 17-kDa proteins, or spermadhesins, that are not found in epididymal sperm. These proteins are heparin-binding proteins [1,2] and are peripherally associated with the spermatozoon. Removal of these proteins appears to be a prerequisite for the AR [3]. Cross [4] also described an inhibitory effect of seminal plasma on sperm capacitation and identified this inhibitory activity as that of cholesterol. Evidence for sterol depletion during in vivo [5] and in vitro capacitation has been obtained [6]. Moreover, sperm cholesterol plays an important role in controlling the development of acrosomal responsiveness to progesterone in vitro [7] or to the calcium/proton exchange ionophore, ionomycin [8]. Cholesterol depletion in the acrosomal and postacrosomal regions may be a requirement for the initiation of the AR and sperm-egg binding. Similarly, changes in the cholesterol level in the plasma membrane over the midpiece and principal piece may increase lateral mobility of the membrane components, supporting hyperactivated motility for penetrating an egg [9].

A time-dependent cholesterol removal from spermatozoa is observed in the presence of serum, which has a beneficial effect on capacitation and fertilization as has been observed for ovine in vitro fertilization [10]. Several cholesterol acceptors have been tested in vitro, albumin being one of the most prominent proteins supporting in vitro capacitation by accepting cholesterol. Sterol depletion by albumin is highly dependent on the phospholipid content in the type of albumin used. Commercial preparations of serum albumin contain variable amounts of fatty acids and other contaminants, which prevent albumin from manifesting its full steroid-binding affinity. Through elimination of BSA contaminants, AR is enhanced [11].

Components that act as cholesterol acceptors are present in the female tract, the major protein in the uterus and oviduct being albumin. Because of the long residency of spermatozoa in the isthmus prior to ovulation, the oviduct is considered the principal site for completion of sperm capacitation. High-density lipoproteins present in bovine oviductal secretions appear to support cholesterol efflux from bovine spermatozoa [12]. Follicular fluid, also present at the site of fertilization, may promote spontaneous human sperm AR, although by nonspecific induction, as described by Mortimer and Camenzind [13]. All these biological fluids have been used for supporting in vitro capacitation and AR.

Other newly discovered nonphysiological cholesterol acceptors have been used to alter the membrane cholesterol content in several cell types. Three different cyclodextrins (α-, β-, and γ-cyclodextrin) have been used to alter the lipid composition of erythrocytes. β-Cyclodextrin (βCD), a cyclic oligosaccharide consisting of 7 β(1-4)-glucopyranose units, was found to selectively extract cholesterol from the plasma membrane in preference to other membrane lipids [14]. Cyclodextrins enhance the solubility of nonpolar substances by incorporating them in their hydrophobic cavity and forming inclusion complexes. The very high efficiency of cyclodextrins in stimulating cholesterol efflux [15] makes them valuable in studying the influence of cholesterol on membrane protein function. Modification of the cholesterol content of the isolated plasma membranes has been used to study the effect of cholesterol on the binding function of the myometrial oxytocin receptor [16]. These results suggest a direct interaction between the oxytocin receptor and cholesterol, inducing a high-affinity state in the receptor. Although βCD is not a biological molecule found in the female reproductive tract or in oocyte envelopes, it can be used as a highly efficient cholesterol acceptor to investigate the role of cholesterol release as an early event of in vitro sperm capacitation and AR. We pre-

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sent a study of the kinetics of both cholesterol efflux and AR in the presence of βCD.

MATERIALS AND METHODS

Chemicals

The following chemicals were used: β-cyclodextrin and glutaraldehyde (Fluka, Madrid, Spain); BSA (fraction V, fatty acid free; Boehringer Mannheim, Mannheim, Germany); heparin (Rovi, Madrid, Spain), cholesterol (ICN, Aurora, OH); chloroform and methanol (Scharlau, Barcelona, Spain). M-199 H incubation medium was from Biological Industries, Beit Haemek, Israel. All other chemicals were obtained from Sigma (Alcobendas, Spain).

Sperm Preparation

Spermatozoa from the goat (*Capra irca*) were obtained from two fertile males by artificial vagina technique. The quality of the sample was checked before every experiment. Spermatozoa were washed three times in M-199 incubation medium and centrifuged at 300 × *g* for 10 min to remove seminal plasma. The number of spermatozoa in the ejaculate was counted by means of a Neubauer chamber and divided into 2-ml aliquots (8×10^7 cells/ml). One aliquot of washed goat spermatozoa was separated without any treatment and used as a control at time zero. The other aliquots were incubated under capacitation conditions, 37°C, 10% CO_2, in M-199 medium for various times in the presence or absence of βCD, as cholesterol acceptor, at several concentrations. Alternatively, the following components were dissolved in M-199 medium: 20 μg/ml heparin; 10 mg/ml BSA; 10 mg/ml BSA+20 μg/ml heparin; 8 mM βCD; 8 mM βCD+20 μg/ml heparin. The incubation process was stopped by addition of 8 ml Tris-buffered saline, pH 7.4 (TBS), centrifuged, and washed twice by resuspension in the same buffer.

Different determinations were performed in parallel for each sperm suspension after incubation. For lipid analysis, 150×10^6 cells were taken, and centrifugation was performed at 3300 × *g* for 10 min to ensure quantitative recovery of cells; the last pellet was resuspended in 0.5 ml TBS and stored at −20°C until analyzed. For acrosomal status and vitality measurements, 10×10^6 cells were taken; centrifugation was performed at 1000 × *g* for 10 min, to avoid cell damage, and the last pellet was resuspended in 50 μl TBS.

Analytical Procedures

Lipids were extracted both from intact and incubated spermatozoa using a modification of the method of Bligh and Dyer [17] as described by Wolf et al. [18]. The concentration of unesterified cholesterol was determined in the lipid extracts from 100×10^6 spermatozoa by a modification of a commercially available enzymatic serum cholesterol assay (HDL Cholesterol; BioSystems, Barcelona, Spain), which was free of cholesterol esterase (EC 3.1.1.13). Briefly, cholesterol oxidase (EC 1.1.3.6) oxidizes free cholesterol, but not cholesterol esters, to cholest-4-en-3-one with H_2O_2 release. The peroxide oxidatively couples with 4-aminoantipyrine and dichlorophenolsulfonate in the presence of peroxidase (EC1.11.1.7) to yield quinoneimine, a chromogen with maximum absorption at 500 nm [19]. Dried lipid extracts were used as the material to be analyzed. To ensure solubilization of the lipid extracts during the assay procedure, detergent Triton X-100 was added to the Reagent A solution (35 mM phosphate, 0.5 mM sodium cholate, 4 mM dichlorophenolsulfonate, pH 7.0) to a final concentration of 7.7 mM [20]. We estimated that the lowest level of sensitivity for this cholesterol assay was around 1 μg.

Phospholipids were measured colorimetrically in the lipid extract of 50×10^6 spermatozoa according to the procedure of Stewart [21]. Since sensitivity of this method differs depending on the phospholipid headgroups [22], this assay was used only to monitor possible changes in total phospholipid content. In the case of dipalmitoyl lecithin, the lowest sensitivity level was 5 μg [21]. Sperm cholesterol and phospholipid content were expressed per number of spermatozoa and were normalized within each experiment with respect to the cholesterol or phospholipid content of the 0-h control (100%). The results were expressed as percentages with respect to the control.

Acrosomal Status and Vitality

AR and vitality were measured by the triple-stain technique of Talbot and Chacon [23]. Briefly, the sample was incubated with 1% trypan blue for 15 min at 37°C to evaluate sperm vitality. Sperm were washed for removal of excess stain and fixed in 1.5% glutaraldehyde for 15 min. After two washing cycles, sperm were stained with Bismarck Brown and Bengale Rose. We observed the acrosomal status under a Zeiss AxioPlan (Carl Zeiss, Thornwood, NY) epifluorescence microscope after washing. Approximately 400 spermatozoa were scored for each sperm preparation, the samples being scored randomly. The advantage of this technique is that live acrosome-reacted spermatozoa are distinguishable from dead spermatozoa with degenerative acrosomal loss. To validate the triple-stain technique, we compared the results obtained by this technique and the lectin *Pisum sativum*-fluorescein isothiocyanate technique [24]. There were no significant differences in the percentage of AR (AR%) as measured by the two techniques.

Statistical Analysis

Statistical analyses were performed with the SPSS for Windows software package (version 7.5.2S; SPSS Inc., Chicago, IL; 1997). The mean comparison was performed by ANOVA (the a posteriori method of Gabriel was used to compare the mean values of different samples, and Dunnett's method was used to compare each sample with the control). The sample size, n, was 3 to 6. Significance was indicated by $P < 0.05$ or $P < 0.01$.

RESULTS

Phospholipid and unesterified cholesterol content were measured on untreated spermatozoa. These were the control values used as reference for all treatments. The results were as follows: unesterified cholesterol was 8.2 ± 0.5 μg/10^8 spermatozoa (mean ± SEM, n = 6), and total phospholipid was 46 ± 2 μg/10^8 spermatozoa (mean ± SEM, n = 6). After goat sperm incubation with βCD, the release of cholesterol was measured by means of the cholesterol content in spermatozoa. Cells were treated for 150 min with increasing amounts of βCD (from 0 mM to 16 mM). As shown in Figure 1, a very effective concentration-dependent cholesterol efflux was observed. The curve displays a hyperbolic-like saturation shape. At very low concentration of βCD (2 mM), the loss of cholesterol was around one

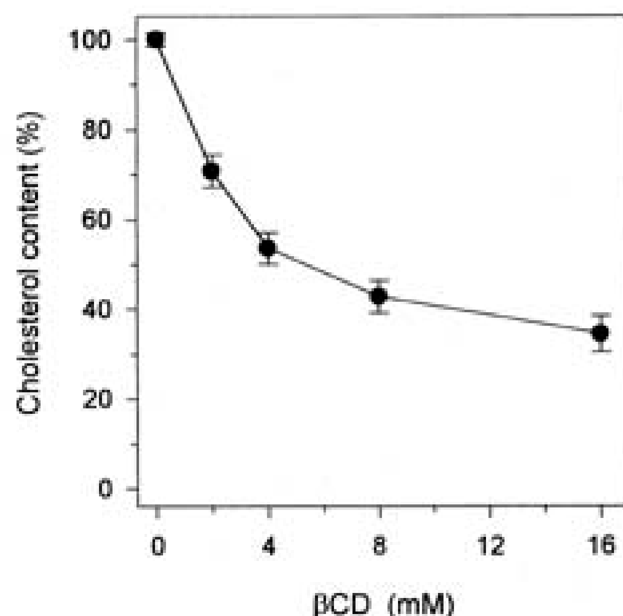

FIG. 1. Dose-response relationship between the βCD content in the medium and the percentage of unesterified cholesterol remaining in spermatozoa after 150-min incubation. Cholesterol content was normalized within each experiment with respect to the cholesterol content of the 0-h control sample (100%). Cholesterol content at 2 mM βCD and higher concentrations was significantly ($P < 0.05$) less than in the control. There were no significant ($P > 0.05$) differences between the cholesterol content at 8 mM and 16 mM βCD. Data are expressed as mean ± SEM (n = 4).

half of the total loss. Maximal efficiency, that is, 65% cholesterol removal, was obtained at 16 mM βCD. After the same incubation time, no significant ($P > 0.05$) removal of phospholipid was observed at any βCD concentration (data not shown).

The βCD-induced AR was also studied after 150-min

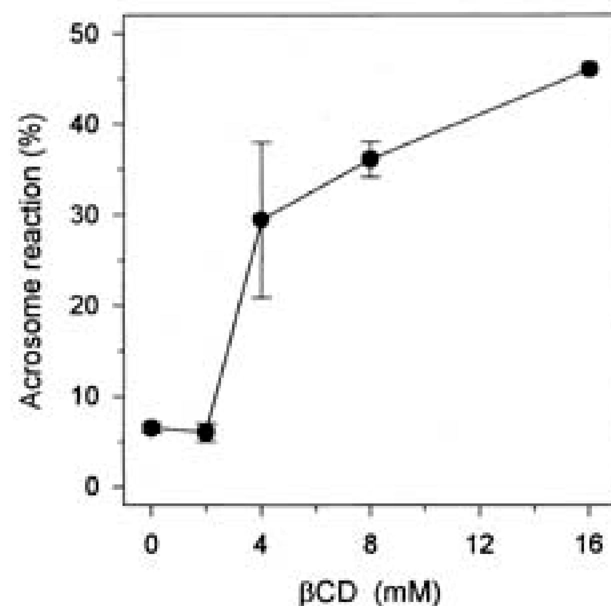

FIG. 2. Dose-response relationship between the βCD content in the medium and the AR%. Goat spermatozoa were incubated in M-199 medium containing different concentrations of βCD. After 150 min, acrosomal responsiveness was determined. The AR% showed a significant ($P < 0.05$) increase at βCD concentrations ≥ 4 μM. Data are expressed as mean ± SEM (n = 4).

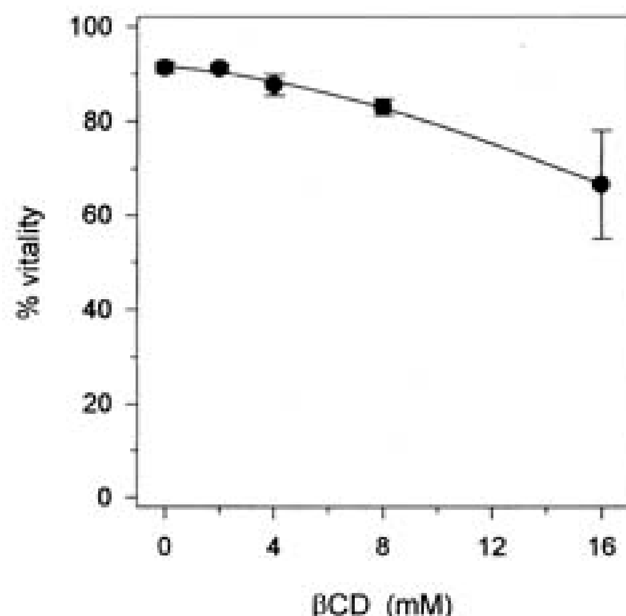

FIG. 3. Assessment of goat sperm vitality after 150 min in M-199 incubation medium containing different concentrations of βCD. Sperm vitality remained very high throughout the experiment but significantly decreased at 8 mM βCD ($P < 0.05$) and at 16 mM βCD ($P < 0.001$). Data are expressed as mean ± SEM (n = 4).

incubation of sperm with increasing concentrations of βCD, from 2 mM to 16 mM (Fig. 2). In contrast to the hyperbolic saturation curve displayed by cholesterol desorption, the AR% displayed a sigmoidal dependence on cyclodextrin concentration. At concentrations below 2 mM, the AR percentage was extremely low, whereas it rose steeply at higher concentration.

To test the possible toxic effect of βCD on sperm, the percentage of vitality was assessed by trypan blue staining after the 150-min incubation period. Samples showed high vitality percentages ranging between 83% and 97% for all the βCD concentrations between 0 and 8 mM (Fig. 3). Noticeably decreased vitality (68%) appeared at 16 mM βCD. Therefore, 8 mM was taken as the optimal βCD concentration to be used in the kinetic study of cholesterol removal and AR induction.

βCD was added at a concentration of 8 mM βCD to the incubation medium (M-199) to study its efficiency on cholesterol removal in terms of time dependency. In Figure 4 we show the cholesterol remaining in sperm membranes after this treatment. Cholesterol efflux, analyzed as described above, displayed exponential kinetics with a half-time of around 10 min. The effect of 8 mM βCD was to promote cholesterol efflux up to 47%. In contrast, sperm phospholipid content after different times of incubation in 8 mM βCD was not significantly different ($P > 0.05$) from the control level (data not shown).

When we analyzed the percentage of acrosome-reacted cells in the presence of 8 mM βCD, we observed a roughly sigmoidal curve, with no significant change in the AR% during the first 60 min and a steep change in the slope of the curve around 90 min of incubation (Fig. 5). Interestingly, there was no significant change in the cholesterol levels between 60 min and 180 min of incubation (Fig. 4).

In order to understand the mechanism by which βCD promotes both a rapid cholesterol efflux and an increase in the AR%, we investigated the effect of the presence of heparin in the sperm incubation medium. Heparin is con-