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Membrane fluidity changes in goat sperm induced by cholesterol depletion using beta-cyclodextrin

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Abstract

Cholesterol efflux from membranes promotes acrosome reaction in goat spermatozoa. In 1 h of incubation of sperm in the presence of beta-cyclodextrin (βCD), all the interchangeable cholesterol is desorbed from sperm membranes, although acrosome reaction is fully accomplished only after 3–4 h of incubation, as previously published. In the present paper we investigate the effect of cholesterol removal from mature goat spermatozoa on the overall membrane "fluidity" of live cell membranes and of liposomes from sperm lipid extracts. Using steady state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH), we studied the average thermotropic behaviour of membrane lipids, after incubation of live sperm for 1 h in BSA-free medium with the presence/absence of 8 mM β-cyclodextrin, as a cholesterol acceptor. Unimodal and bimodal theoretical sigmoids fitted best to the experimental thermotropic profiles of liposomes and whole cells, respectively. In the case of whole sperm, two phase transitions, attributable to different lipid domains, were clearly separated by using the fitting parameters. After cholesterol removal, important changes in the relative anisotropy range of the two transitions were found, indicating an increase in the "fluidity" of some of the lipid microdomains of sperm membranes. These changes in sperm lipid dynamics are produced before the onset of sperm acrosome reaction.

Keywords: Goat sperm capacitation; Cholesterol efflux; Cyclodextrin; Fluorescence anisotropy; Membrane fluidity

1. Introduction

Sperm capacitation is required to fertilize an egg. During capacitation, spermatozoa are hyperactivated and their acrosome becomes modified in preparation for the acrosome reaction (AR). Incubating spermatozoa in the continued presence of seminal plasma prevents capacitation and AR. Cross [1] described an inhibitory effect of seminal plasma on sperm capacitation and identified this inhibitory effect as being due to cholesterol.

Sperm cholesterol removal is one of the events that has been implicated in the process of capacitation, that renders sperm acrosomally responsive [2]. Evidence for sterol depletion during in vivo [3] and in vitro [4] capacitation has been obtained. Moreover, sperm cholesterol plays an important role in controlling the development of acrosomal responsiveness to progesterone in vitro [5] or to the calcium/proton exchange ionophore, ionomycin [6]. As has been reported, cholesterol depletion in certain domains (acrosomal and postacrosomal regions) of the plasma membrane may be a requirement for the initiation of AR and sperm–egg binding ability [7–10].

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 [11]. Several cholesterol acceptors have been tested in vitro, albumin being one of the most prominent proteins supporting in vitro capacitation [12]. Newly discovered nonphysiological cholesterol acceptors have been used to alter

Abbreviations: AR, acrosome reaction; βCD, β-cyclodextrin; DPH, 1,6-diphenyl-1,3,5-hexatriene; ΔH_{vH}, van't Hoff enthalpy; M-199, Hanks Salts Base incubation medium; PC, phosphatidylcholine; PE, phosphatidylethanolamine; *r*, fluorescence anisotropy; SPM, sphingomyeline; TBS, Tris-buffered saline; T_m, phase transition temperature

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the membrane cholesterol content in several cell types. β-cyclodextrins, cyclic oligosaccharides consisting of 7 β(1–4)-glucopyranose units, were found to selectively extract cholesterol from the plasma membrane, in preference to other membrane lipids [13]. Several authors have used β-cyclodextrin (βCD) or its derivatives to induce cholesterol removal from sperm and to study the role of cholesterol efflux in promoting acrosome reaction (AR) [10,14–19]. Yancey et al. [20] demonstrated that the order of efficiency in accepting cholesterol is methyl-β-cyclodextrin (MβCD)>2-hydroxypropyl-β-cyclodextrin (2OHpβCD)>β-cyclodextrin (βCD). Although these are not biological molecules found in the female reproductive tract or in oocyte envelopes, they have been used as highly efficient cholesterol acceptors to investigate the role of cholesterol release as an early event of in vitro sperm capacitation and AR.

As we previously demonstrated, 30 min after the addition of βCD to goat sperm, a final removal of about 47% of the total cholesterol contained in sperm membranes is obtained [10]. Despite this rapid cholesterol removal, the acrosome is not responsive for 60 min. Interestingly, membrane lipid dynamics may play a role in the onset of acrosome reaction. We reported an exponential decay of cholesterol, in contrast with the sigmoidal kinetics of acrosome reaction from 1 h to 3–4 h after the addition of βCD to the cell suspension.

Therefore, we were interested in following the changes in the lipid dynamics of sperm membranes induced by cholesterol depletion during the initial 60 min of incubation, and before the onset of AR. As cholesterol depletion has been related to changes in membrane fluidity [4,18,21–25], we investigated these changes in sperm membranes using fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH), which gives information on the average physical state and lipid domain organization of live sperm membranes. This study with whole goat sperm will contribute to better define the biochemical–biophysical parameters of sperm capacitation. Our results on liposomes built with goat sperm lipid extracts will allow a comparative study with several data on liposomes obtained from mammalian spermatozoa reported in the literature.

2. Materials and methods

2.1. Chemicals

The following chemicals were used: β-cyclodextrin (βCD) (Fluka, Madrid, Spain), cholesterol (ICN, Aurora, Ohio, USA), chloroform and methanol (Scharlau, Barcelona, Spain), 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes, Leiden, The Netherlands), dry N_2 (Air Liquide España S. A., Madrid, Spain). M-199 (with Earle's Salts and L-Glutamine) and M-199 (with Earle's Salts and L-Glutamine but without Phenol Red) incubation media were from Gibco Invitrogen, Barcelona, Spain. All other chemicals were obtained from Sigma (Alcobendas, Spain).

2.2. Sperm preparation

Goat (*Capra hircus*) spermatozoa were obtained from two fertile males using the artificial vagina technique. The quality of the sample was checked before every experiment. Spermatozoa were washed three times in M-199 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. The percentage of motile sperm was estimated by using a phase-contrast microscope. Sperm viability was assessed by using a nucleic acid stain, Hoechst 33258, and visualized by epifluorescence microscopy [26].

2.3. Cholesterol removal from spermatozoa

The optimal conditions for a significant goat sperm cholesterol efflux with a negligible acrosome reaction were established in a previous publication [10]. Accordingly, aliquots with 8×10^7 cells were incubated here for 1 h in capacitation conditions, at 37 °C, 10% CO_2, in M-199 medium in the presence/absence of 8 mM β-cyclodextrin (βCD) as cholesterol acceptor. Motility and viability of 8 mM βCD-treated sperm were also evaluated as described in Section 2.2.

Two samples were used for fluorescence anisotropy studies: intact whole cells or liposomes prepared from sperm lipid extracts. When using intact sperm, the incubation was stopped by diluting 5/1 with M-199 medium without Phenol Red, followed by centrifugation in mild conditions (300×*g* for 10 min), and washing twice in the same medium. In the case of sperm samples that were used to obtain lipid extracts, the incubation process was stopped by diluting 5/1 with TBS buffer (10 mM Tris, 0.15 M NaCl) pH 7.4, centrifuged at 3500×*g* for 10 min, to ensure quantitative recovery of cells, and washed twice by resuspension in the same buffer; the last pellet was resuspended in a small volume (less than 1×10^9 cells / ml) of TBS buffer and stored at −20 °C until lipid extraction.

2.4. Lipid extraction from sperm

Lipids were extracted with organic solvents both from control and βCD-treated spermatozoa using a modification of the method of Bligh and Dyer [27] according to Wolf et al. [28]. Lipid extracts were stored at −20 °C until liposome preparation or cholesterol determination.

2.5. Cholesterol determination

Dried lipid extracts were used as the material to be analyzed. The concentration of unesterified cholesterol was determined in the lipid extract from 1×10^8 spermatozoa, using a modification of a commercially available enzymatic serum cholesterol assay (HDL Cholesterol; BioSystems, Barcelona, Spain) which was free of cholesterol esterase (EC3.1.1.13), as described previously [10]. Briefly, cholesterol oxidase (EC 1.1.3.6.) oxidizes free cholesterol and some other naturally-occurring β-hydroxy sterols, 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 (Ec 1.11.1.7) to yield quinoneimine, a chromogen with maximum absorption at 500 nm [29]. 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.

2.6. Liposome preparation

Multilamellar liposomes (MLVs) were prepared from organic solvent lipid extracts of control or βCD-treated spermatozoa. The solvent was evaporated under an oxygen-free N_2 stream and the resulting film was maintained under high vacuum overnight to remove organic solvent traces. Vesicles were obtained by resuspending the film with TBS buffer by vortex mixing. The suspensions were then homogenized by sonication for 30 s in a Dynatech Sonic Dismembrator (New York, USA) and kept for 1 h at 37 °C under magnetic stirring.

2.7. Labeling with fluorescent probe DPH

The fluorescent lipophylic molecule 1,6-diphenyl-1,3,5-hexatriene (DPH) has been established as a probe to detect the "fluidity" of the lipid bilayer of liposomes, biological membranes and whole cells, by monitoring the anisotropy