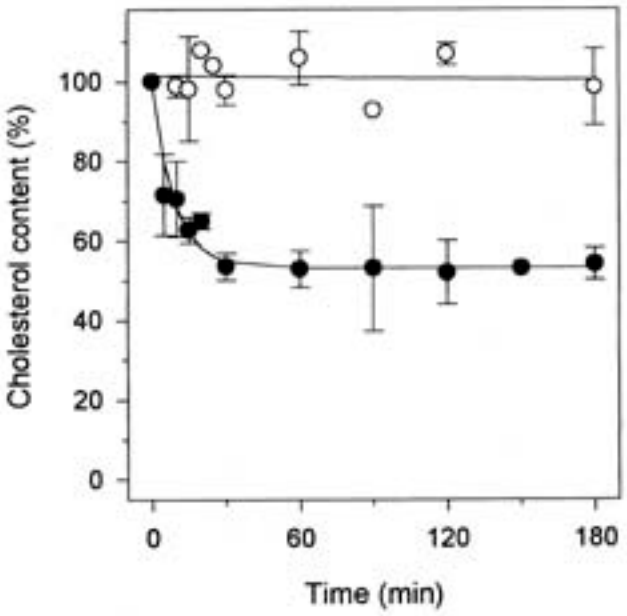

FIG. 4. Kinetics of the removal from goat spermatozoa of unesterified cholesterol induced by 8 mM βCD (filled circles). Cholesterol content was normalized within each experiment with respect to the cholesterol content of the 0-h control sample (100%). Cholesterol levels in control samples at different times of incubation without βCD (empty circles) are included for comparison. All sperm samples incubated with βCD showed a significant loss of cholesterol, as compared with the control for the same incubation time: 5 min ($P < 0.05$) or 10–180 min ($P < 0.01$). Data are expressed as mean ± SEM (n = 3).

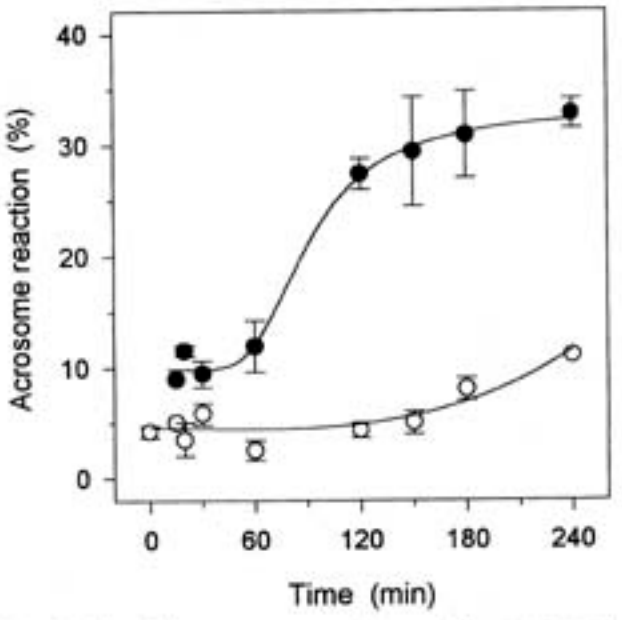

FIG. 5. Kinetics of the acrosome responsiveness of live goat spermatozoa after incubation in 8 mM βCD (filled circles) or in medium without βCD (open circles). All the values for incubation times ≥ 60 min were significantly ($P < 0.05$) higher than in the corresponding controls. There was no significant difference ($P > 0.05$) either between 10, 20, 30, and 60 min or between 120, 150, 180, and 240 min. The $t_{1/2}$, or inflexion point, of the sigmoidal curve was 94 min ± 12 min. Data are expressed as mean ± SEM (n = 3).

sidered by several authors to be an inducer of the AR through removal of coating proteins from the sperm membrane surface [25,26]. In our experiments, incubation of goat spermatozoa in the presence of 20 μg/ml heparin for 2 h was unable to induce AR (Fig. 6). On the other hand, the coincubation of goat spermatozoa with 10 mg/ml albumin and 20 μg/ml heparin showed a synergistic effect on the AR%, although the cholesterol content did not change. Both AR and membrane cholesterol removal were increased in the sole presence of βCD. The coincubation of spermatozoa with 8 mM βCD and 20 μg/ml heparin, however, did not significantly modify the effect of βCD alone. Thus, βCD was able both to remove cholesterol from sperm membranes and to promote AR without the cooperation of heparin.

DISCUSSION

Evidence from several sources supports the idea that cholesterol efflux may be important in capacitation, but it is not well established whether major removal of cholesterol from the membrane might itself induce the AR. Our aim in the present work was to evaluate the cholesterol efflux induced by an effective cholesterol acceptor molecule and to study the extent to which it is related to the induction of the AR.

Albumin has been used extensively in the incubation medium for an in vitro induction of the AR [27–31], although the results are very controversial. Using albumin, some authors obtained cholesterol efflux [27,32,33] but others did not [31,34]. It may be worth considering the origin of the albumin used in the experiments. Commercial albumin has several impurities, and among them there are lipids. When the albumin source is serum or other biological fluids, inducers of the AR are present. This may explain

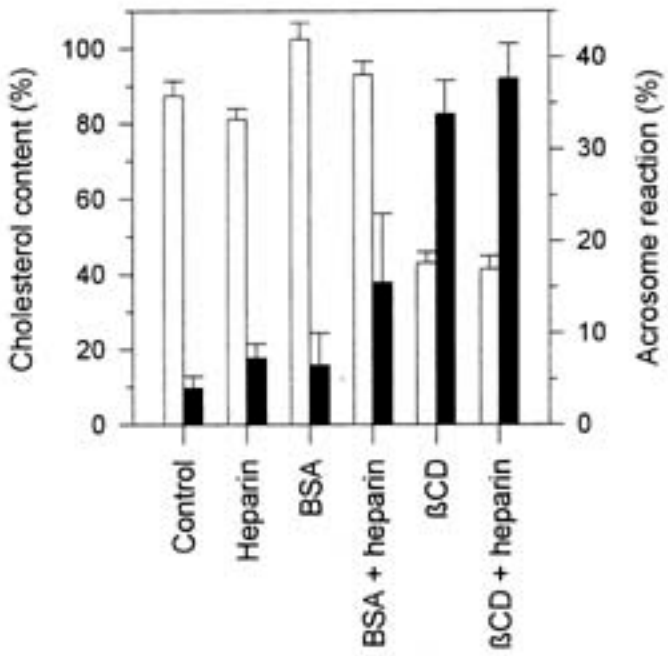

FIG. 6. Effect of 150-min incubation of goat spermatozoa in various media on unesterified cholesterol content (open bars) and on acrosomal responsiveness (solid bars). Cholesterol content is expressed as percentage of the value in M-199 medium and t = 0. Cholesterol content at 8 mM βCD and 8 mM βCD+20 μg/ml heparin was significantly ($P < 0.05$) less than in other conditions, but there was no significant ($P > 0.01$) difference in cholesterol content between 8 mM βCD and 8 mM βCD+20 μg/ml heparin. The AR% showed a significant ($P < 0.05$) increase at 8 mM βCD and at 8 mM βCD+20 μg/ml heparin. There was no significant ($P > 0.01$) difference in AR% between 8 mM βCD and 8 mM βCD+20 μg/ml heparin. Data are means ± SEM (n = 3).

the broad range of cholesterol efflux percentages described in the literature.

It has been reported that extensive release of cholesterol from erythrocyte membranes can be achieved upon incubation of the cells with βCD. There was a rapid cholesterol exchange (< 1 min) [35] from erythrocytes to βCD, which is in contrast with the cholesterol transfer from these cells to serum or isolated lipoproteins (1–8 h) [36]. The very fast exchange rate of βCD makes this molecule highly useful as a cholesterol acceptor in capacitating medium.

Our results indicate that at 2 mM βCD, 30% of cholesterol was released from sperm membranes, but this concentration did not induce any AR. Only when more cholesterol was lost were membranes susceptible to initiating all the changes leading to capacitation and AR. When cholesterol efflux had increased to 50%, at a concentration of 4 mM βCD, we observed more than half of the maximal AR percentage. Cholesterol removal between 50% and 65% was able to induce the AR. The use of concentrations of βCD higher than 8 mM resulted in only a moderate increase in cholesterol efflux and AR%. Our results suggest that there is a direct effect of cholesterol efflux on the AR induction rather then any kind of binding of βCD to membranes. In fact, some authors have demonstrated in erythrocytes that cholesterol removal by βCD occurs with negligible binding to the cell membrane [14]. That is, βCD extracts cholesterol from membranes into a new compartment located in the aqueous phase, while phospholipid levels remain unchanged. This agrees with the fact that there was no membrane loss from spermatozoa, so we could consider only the cholesterol efflux.

The sigmoidal shape of the curve (Fig. 2), indicating the AR% versus βCD concentration, can be interpreted as a cooperative effect in which the early molecular events in a restricted area of sperm membrane may facilitate further changes in a broader zone, thus inducing a quick AR in a short period of time.

Cholesterol efflux kinetics due to βCD ($t_{1/2}$ around 10 min) is much more rapid than the reported kinetics ($t_{1/2}$ > 1 h) of sperm membrane cholesterol efflux in the presence of estrous sheep serum [32] or human female serum or follicular fluid [10]. Small-particle cholesterol acceptors show more rapid kinetics of cholesterol uptake from cells than macromolecular acceptors [37]. This rapid loss of cholesterol from sperm membrane, which follows an exponential decay (Fig. 4), contrasts with the S-shaped kinetics of AR increase (Fig. 5). After 30 min of incubation in the presence of 8 mM of βCD, all the exchangeable cholesterol has been released from the membranes, but a negligible AR is obtained. Incubation for 1–2 h is required to reach the maximal AR value. Cholesterol efflux has been proposed as an early event previous to the AR [7,27,38,39]. Our kinetic study of both cholesterol efflux and AR suggests that acrosome responsiveness is triggered by a major cholesterol loss.

Several authors have reported significant cholesterol removal through addition of various components to the incubation medium or through use of biological fluids. Huneau et al. [10] obtained a time-dependent cholesterol removal ranging from 54% to 67% after 5 h of incubation in DM-H medium containing 20% estrous sheep serum, whereas removal was limited to 14 ± 3% in the absence of serum. Female biological fluids have been extensively used, and similar cholesterol efflux was obtained. Langlais et al. [32] incubated spermatozoa for 4 h at 37°C in Ham's F-10 medium supplemented with human female serum or follicular fluid and obtained cholesterol efflux in a time-dependent manner (57% and 42%, respectively). With spermatozoa incubated in protein-free medium, low cholesterol efflux (14%) occurs.

The role of albumin in the uptake of cholesterol has been discussed. There is some evidence that albumin impurities can modify the ability of albumin to bind cholesterol. For Langlais et al. [32], after a period of 2-h incubation with 3.5% HSA (fraction V, Calbiochem-Boehring; La Jolla, CA) the uptake of cholesterol was 38% versus 12% for cells incubated in medium alone. In contrast, using similar concentrations of albumin, others found that cholesterol efflux from sperm membranes was not statistically significant [31]. As shown by Sugkraroek et al. [34], the cholesterol level from fertile donors' sperm declines < 5% when incubated in BWW medium supplemented with 3.5% HSA for 5 h. Even after long periods of incubation (18–20 h), Benoff et al. [28] obtained a cholesterol decrease of only 15% in swim-up spermatozoa incubated in Ham's F-10 with 30 mg/ml HSA.

Interestingly, the sample some studies used was ejaculated spermatozoa [31,32,34], whereas others used epididymal spermatozoa [27,33]; this would confer on the sample a differential accessibility for cholesterol acceptors, probably because of the presence of coating proteins in ejaculated spermatozoa. At low concentrations of albumin in the medium, a relatively high amount of cholesterol could be removed from the membranes of epididymal spermatozoa, these lacking all the coating molecules that are adsorbed from seminal plasma molecules. Go and Wolf [27] obtained a 20–59% decrease in mouse epididymal sperm cholesterol with several commercially purified BSA fractions (20 mg/ml). Davis et al. [33] also gave evidence of a 12% cholesterol transfer from cauda epididymal rat sperm to albumin after incubation in 4 mg/ml BSA for 5 h.

Ehrenwald et al. [40] did not detect any acrosome-reacted spermatozoa after 31% cholesterol efflux in 90 min. They obtained acrosome responsiveness only after the addition of lysophosphatidylcholine. Our results show that if insufficient cholesterol is removed from the membrane (around 30%), as detected in 2 mM βCD, no AR is induced. However, if 40–60% of cholesterol was removed, as occurs in 4 mM βCD, we observed that AR was induced.

It has been determined both in vivo and in vitro that heparin or glycosaminoglycans capacitate sperm, probably due to their ability to sequester coating proteins, some of them, for this reason, named heparin-binding proteins [25,40]. We do not find a synergistic effect on the cholesterol efflux or on the AR percentage when adding heparin to βCD. This could indicate that the removal of coating proteins is not necessary for cholesterol uptake by βCD.

In our study we provide evidence that with use of βCD in the medium, a high percentage of cells undergo the AR, the magnitude of the response being in the range of the induced AR obtained by other authors with different inducers. Cross [8] reported 30% AR in human spermatozoa when induced with progesterone in the presence of 26 mg/ml of BSA in the medium. Alternatively, a fusogenic lipid, lysophosphatidylcholine (LC), may be used in the capacitation medium as a potent inducer of AR. With use of LC, as much as an 80% increase in the percentage of acrosome-reacted cells was obtained by Parrish et al. [25], but spermatozoa in which the AR is not induced by LC achieve only a 15% spontaneous AR among the sperm population. Our results might suggest that merely removing sufficient

cholesterol from sperm membranes would trigger a series of molecular events leading to AR.

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