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Cholesterol Inhibitory Effects on Human Sperm–Induced Acrosome Reaction

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ABSTRACT: Progesterone (P_4) is known to induce an acrosome reaction in mammalian sperm in vitro, whereas cholesterol is a major inhibitor of acrosome reaction. This study had three objectives: to study the in vitro effects of exogenous cholesterol on acrosome reactions in human sperm, to study the mechanism by which cholesterol affects P_4-induced acrosome reaction and those induced by dibutyryl cyclic adenosine monophosphate (db-cAMP), and to study the status of the P_4 surface receptor during capacitation and acrosome reaction and its relationship with cholesterol and different acrosome reaction inducers. Acrosome reaction was induced with exposure to 10 μg/mL of P_4 for 30 minutes and 1 mM of db-cAMP for 30 minutes in motile sperm either in the presence or absence of 0.1–1 μg/mL of cholesterol for 30 minutes. The effects of a 30-minute exposure to 1 μg/mL of β-sitosterol, a cholesterol plant analogue, as well as the effects of cholesterol on P_4-induced acrosome reactions were compared. Fluorescein isothiocyanate-labeled albumin-progesterone conjugate (P_4-FITC-BSA) was used as the probe in order to quantify the percentage of sperm in which the P_4 surface receptor was exposed. The results of this study indicate that cholesterol inhibited P_4-induced acrosome reactions when added to the sperm during capacitation (long incubation) and when it was added with P_4 during the induction of acrosome reactions (short incubation). Similarly, acrosome reaction that was induced by db-cAMP was also inhibited by cholesterol. Fifty percent of P_4-induced acrosome reaction was inhibited by a cholesterol concentration of 0.2 μg/mL. Cholesterol's inhibition of induced acrosome reaction was independent of P_4 concentration. β-Sitosterol inhibited P_4-induced acrosome reaction in a dose-dependent manner that was identical to that of cholesterol. We observed that increases in the P_4 surface receptor exposure were time-dependent and receptors migrated toward the equatorial segment during the first 2 hours of capacitation. We also found that db-cAMP induced the appearance of the P_4 surface receptor in the sperm plasma membrane and that cholesterol inhibited it. The results of this study suggest that cholesterol inhibits acrosome reaction in a noncompetitive manner by modifying the structure of the sperm plasma membrane, which prevents exposure of the P_4 surface receptor for P_4 binding.

Key Words: Surface receptors, signal transduction pathways, β-cyclodextrin, modified human tubal fluid.

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Several studies since 1988 have demonstrated the effects of progesterone (P_4) on sperm functions, particularly the effect that P_4 has in facilitating an intracellular calcium influx, which results in an acrosome reaction (Osman et al, 1989; Thomas and Meizel, 1989; Blackmore et al, 1990; Baldi et al, 1991; Parinaud et al, 1992; Sabeur and Meizel, 1995; Doherty et al, 1995). However, the precise role of P_4 on the signal transduction pathways of acrosome reaction is not completely understood, although interactions between P_4 and its receptors on the sperm surface have been determined (White and Aitken, 1989; De Jonge et al, 1991; Rotem et al, 1992; Furuya et al, 1993; Bielfeld et al, 1994). Recent studies suggest that acrosome reaction induced by P_4 involve both an early and a latent calcium influx in human spermatozoa by respectively triggering the opening of a calcium channel on the plasma membrane and activating protein–tyrosine kinase (PTK) (Luconi et al, 1995; Tesarik et al,1996).

The lipid composition of the sperm plasma membrane is different from that found in somatic cells (Poulos and White, 1973; Mack et al, 1986; Alvarez and Storey, 1995). One of these lipid components is cholesterol, which plays a major role in regulating the fluidity of the lipid bilayer, membrane permeability, the mobility of integral proteins, and functional receptors in the membranes (Benoff, 1993; Langlais and Roberts, 1985). Cholesterol is known to be one of the major components of seminal plasma (Davis, 1976; Zarintash and Cross, 1996; Cross, 1996b, 1997) and has been shown to inhibit human sperm acrosome reaction (Ehrenwald et al, 1988; Benoff et al, 1993; Zarintash and Cross, 1996) and to regulate the acrosome response to P_4 (Cross, 1996, 1997, 1998). However, its underlying mechanism, in particular, the mechanisms of its efffect on P_4-induced acrosome reactions, remain unknown. One possible hypothesis is that cholesterol may alter the sperm plasma membrane's structure or fluidity, thereby exposing P_4 surface receptors for ligand binding and action. Interactions between P_4 and its receptors on the sperm membrane surface have been implicated (White and Aitken, 1989; Bielfeld et al, 1994); however, there is a contradiction among the studies because there are probably different types of P_4 receptors on the sperm plasma membrane.

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In this study, we performed experiments to elucidate the cellular mechanisms of the effects of cholesterol on acrosome reactions induced by P_4 and those induced by db-cAMP. We also studied the effects of cholesterol and db-cAMP on exposure of the P_4 surface receptor and the status of the P_4 surface receptor during sperm capacitation and acrosome reaction.

Materials and Methods

Chemicals

We purchased the following chemicals from Sigma Chemical Company, St Louis, Mo: 2′-o-dibutyryl adenosine 3′:5′-cyclic monophosphate (db-cAMP), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), *Pisum sativum* agglutinin (PSA), fluorescein isothiocyanate (FITC)-labeled PSA, progesterone β-cyclodextrin complex (water-soluble P_4), cholesterol β-cyclodextrin complex (water-soluble cholesterol), β-sitosterol, FITC-labeled albumin (FITC-BSA), and FITC-labeled progesterone–albumin conjugate (P_4-FITC-BSA). Human tubal fluid (HTF) was purchased from Irvine Scientific, Santa Ana, Calif, and was modified by adding 5 mg/mL of human serum albumin (modified HTF) (Biggers et al, 1971; Overstreet et al, 1980).

Semen Samples

Semen samples were provided by fertile donors at the Andrology Laboratory of the University of British Columbia. Ejaculates were obtained by masturbation after at least 72 hours of abstinence and were prepared within 2 hours. All ejaculates demonstrated normal volume, sperm motility, and morphology according to World Health Organization (1992) criteria.

Sperm motility and viability were estimated before and after each treatment. Motility was determined by counting motile and nonmotile spermatozoa in at least 10 separate and randomly selected fields; viability was determined by using the supravital staining technique, which involved staining the spermatozoa first with 1% eosin in distilled water and then with 10% nigrosin in distilled water (WHO, 1992). In each case, at least 100 spermatozoa were counted to estimate motility and viability.

Swim-Up Procedure

After semen samples were liquefied at room temperature, highly motile sperm were recovered by a swim-up method in modified HTF (Biggers et al, 1971; Lopata et al, 1976; Overstreet et al, 1980). Sperm with a motility of greater than 90% were recovered from the top layer of the test tube and were concentrated by centrifugation for 2 minutes (1400 × *g*). The sperm pellet was then resuspended in modified HTF and the final concentration was adjusted to 5×10^6 motile sperm/mL (Biggers et al, 1971; Overstreet et al, 1980). Sperm were then incubated in 100-μL drops of a capacitation medium of modified HTF under mineral oil in a 96-well culture dish for 2 to 18 hours at 37°C and 5% CO_2.

Assessment of Acrosome Status

Changes in acrosome reaction were estimated by direct fluorescence assay (Cross and Meizel, 1989). After each treatment, a 5-μL droplet of sperm solution was uniformly spread onto each well of a slide, air-dried, and fixed with methanol. Each well was individually washed 3 times in a phosphate-buffered solution (+ 0.5% BSA) and stained with FITC-PSA. Sperm were then scored on randomly selected fields until 200 were counted from each well. The acrosome region of acrosome-intact spermatozoa showed a bright fluorescence, whereas the acrosome-reacted spermatozoa did not (Cross and Meizel, 1989). Only spermatozoa that showed a complete and uniform fluorescent pattern of the acrosome region were considered to be acrosome intact. Those that showed an irregular or patchy fluorescent pattern of the acrosome region, a band-like fluorescent pattern on the equatorial segment, or with no fluorescence whatsoever were considered to have been acrosome-reacted. Parallel control experiments under identical conditions were carried out for each set of experiments that examined a particular treatment combination.

Induction of the Acrosome Reaction by Progesterone

Highly motile sperm were prepared for capacitation using a modified HTF medium at 37°C and 5% CO_2 and incubated for 2 to 18 hours. Progesterone (10 μg/mL) was then added to the preparation for 30 minutes to induce an acrosome reaction as described by Lee et al (1998). Acrosome status was assessed using an FITC-PSA probe. At least 200 spermatozoa were counted for each treatment. As a negative control, another group of spermatozoa were prepared under the same experimental conditions but β-cyclodextrin was added to the preparation for 30 minutes.

Effects of Cholesterol on Spontaneous and Progesterone-Induced Acrosome Reactions

To assess the effect of cholesterol on spontaneous acrosome reaction, we incubated sperm with 1 μg/mL of cholesterol in a capacitation medium (modified HTF at 37°C and 5% CO_2) for 0, 2, and 18 hours, after which we assessed acrosomal status. To examine the effect of cholesterol on P_4-induced acrosome reaction, we incubated capacitated sperm with 1 μg/mL of cholesterol for 10 minutes and then exposed them to 1 μg/mL of P_4 for 10 minutes (we referred to this as *short incubation*).

As a parallel experiment, which we referred to as *long incubation,* we prepared another group of spermatozoa with 1 μg/mL of cholesterol in modified HTF, incubated it for 2 to 18 hours, and then added 10 μg/mL of P_4 for 30 minutes.

As a negative control for both sets of experiments, to another group of capacitated sperm preparations we added 10 μg/mL of β-cyclodextrin for 10 minutes (the diluent for P_4 and cholesterol) under identical conditions. At the end of each experiment we assessed acrosomal status using FITC-PSA. At least 200 spermatozoa were counted for each treatment.

Effects of Cholesterol on db-cAMP–Induced Acrosome Reaction

Cyclic AMP (cAMP), which is produced by adenylate cyclase, is a second messenger in the signal transduction pathway that involves protein kinase A (PKA). It is known to induce acrosome reaction in capacitated human sperm (White and Aitken, 1989; De Jonge et al, 1991; Bielfeld et al, 1994). To evaluate

the potential site of cholesterol action, we incubated capacitated sperm for 30 minutes with db-cAMP, which is a more membrane-permeable analogue for the second messenger, cAMP (Bavister and Boatman, 1984). In a parallel treatment, we exposed capacitated sperm to 1 μg/mL of cholesterol for 30 minutes and then added 0.1 mM of db-cAMP for 30 minutes to induce an acrosome reaction. As a negative control, we added 10 μg/mL of β-cyclodextrin for 30 minutes. In both cases, we assessed acrosome status using FITC-PSA. At least 200 spermatozoa were counted for each treatment.

Dose-Dependent Inhibition of Cholesterol on Progesterone-Induced Acrosome Reaction

To further characterize the effects of cholesterol on P_4-induced acrosome reaction, we added different concentrations of cholesterol (0.1, 0.2, 0.3, 0.4, 0.5, and 1 μg/mL in HTF; in each case for 10 minutes) to capacitated sperm before adding 1 or 10 μg/mL of P_4 (in each case for 30 minutes) to induce an acrosome reaction for short incubation experiments. As a negative control, we added β-cyclodextrin alone at concentrations comparable to those of cholesterol and then assessed acrosomal status using an FITC-PSA probe. At least 200 spermatozoa were counted for each treatment.

Dose-Dependent Effect of β-Sitosterol on Progesterone-Induced Acrosome Reaction

To examine the specificity of the effects of cholesterol on P_4-induced acrosome reaction, we chose β-sitosterol (Van Der Kraak and MacLatchy, 1995; Zakaria et al, 1999), which is a plant analogue of cholesterol, to serve as a comparison medium. We treated capacitated sperm with 1 μg/mL of β-sitosterol for 10 minutes and then added either 1 or 10 μg/mL of P_4 for 30 minutes. At each P_4 concentration we assessed the effects of different concentrations of β-sitosterol (0.1, 0.2, 0.3, 0.4, 0.5, and 1 μg/mL in HTF) on the P4-induced acrosome reaction. β-Cyclodextrin or DMSO (the diluent for β-sitosterol with final concentration of less than 1% in sperm solutions) of comparable concentration were used as negative controls, after which we assessed acrosomal status. At least 200 spermatozoa were counted in each treatment.

Time-Dependent Appearance of Progesterone Receptors on Sperm Plasma Membrane

We used fluorescein-labeled P_4-BSA conjugate (P_4-FITC-BSA) as a probe to visualize the P_4 binding sites and to monitor the time-dependent appearance of P_4 receptors on the sperm plasma membrane (Blackmore and Lattanzio et al, 1991). Freshly prepared sperm solutions (10×10^6 sperm/mL) were capacitated in different time intervals that varied from 0 to 240 minutes. P_4-FITC-BSA (100 μg/mL) was then added to the solutions and incubated for 15 minutes in an HTF medium at 37°C in air and 5% CO_2. Spermatozoa were then pelleted by centrifugation ($14\,000 \times g$ for 2 minutes), washed twice in PBS, and smeared on microscopic slides. After drying in air we immediately examined the slides with a fluorescence microscope and assessed the changes in the location of probe binding for at least 200 spermatozoa in each sample. We used FITC-labeled BSA under identical conditions as a negative control probe.

Effects of Progesterone, Cholesterol, and db-cAMP on the Appearance of Progesterone Receptors on Sperm Plasma Membrane

Freshly prepared sperm in a culture medium of 10×10^6 sperm/mL were capacitated for 2 hours and treated with 10 μg/mL of P_4 or 1 mM of db-cAMP either with or without 1 μg/mL of cholesterol as described for the short incubation method. We placed 5-μL droplets of the treatment and control groups on slides and scored the percentage of acrosome reaction and P_4 receptor appearances by fluorescence microscopy after being incubated with FITC-PSA and P_4-FITC-BSA, respectively, to assess any possible relationship. At least 200 spermatozoa were counted in each treatment group.

Assessment of the Presence of the Progesterone Receptor in Methanol-Fixed Sperm

Freshly prepared sperm were washed twice with HTF. Solutions of 10×10^6 sperm/mL were then smeared on 8-well slides and air-dried. After methanol-fixation, we added 25 μL of P_4-FITC-BSA (100 μg/mL) to each well, incubated them for 30 minutes, and washed them twice with PBS. We mounted the slides with 80% glycerol and immediately scored the presence of P_4 receptors using a fluorescence microscope. We counted at least 200 spermatozoa in each treatment.

Statistical Analysis

After each treatment, we assessed the percentages of acrosome-reacted sperm using an FITC-PSA probe and standardized them by subtracting the percentages of sperm that had undergone spontaneous acrosome reaction in concurrent control experiments. We made between-group comparisons by 1-factor analysis of variance and Bonferroni pairwise comparisons when overall F scores corresponded to a P value of less than .05 (statistical significance was defined as $P < .05$). In all experiments, results were reported as the mean values ± the standard error of the mean.

Results

Progesterone-Induced Acrosome Reaction

Similar to a previous observation (Lee et al, 1998), the mean percent changes of acrosome-reacted sperm increased significantly in the presence of P_4 in 8 repeated experiments (Table 1). At a P_4 concentration of 10 μg/mL, the percent change of acrosome-reacted sperm increased significantly by 7.3% ± 1.1% and 10.7% ± 1.2% for sperm that were capacitated for 2 and 18 hours, respectively ($P < .05$ compared with β-cyclodextrin as the control, in which acrosome reaction increased by 1.3% ± 0.5%; Table 1). No significant increases in acrosome-reacted sperm were observed for higher P_4 concentrations or induction times longer than 30 minutes.

Table 1. *Inhibitory effects of cholesterol (CH) on spontaneous and P_4-induced acrosome reaction (AR) (n = 8)**

	Percentage Changes of AR† (Percentage Inhibition)	
	Sperm Capacitation Time	
Chemicals (concentration)	2 h	18 h
Spontaneous (control)	2.2 ± 0.6	5.0 ± 1.2 A
Spontaneous AR + CH (1 μg/mL, 30 minutes)	1.3 ± 0.5	1.3 ± 0.5 B (74.6)
P_4 (10 μg/mL, 30 minutes)	7.3 ± 1.1 A	10.7 ± 1.2 C
P_4 (10 μg/mL) + CH (1 μg/mL) (short incubation)‡	1.3 ± 0.5 C (82.6)	0.6 ± 0.2 D (94.8)
P_4 (10 μg/mL) + CH (1 μg/mL) (long incubation)§	1.9 ± 0.2 C (74.4)	1.9 ± 0.6 D (82.5)

* Values are reported as the mean value ± standard error of the mean.

† Values were the differences between acrosome reaction at time 0 and after each treatment. A indicates values were significantly different from those of spontaneous AR (2 hours), $P < .05$; B, values were significantly different from those of spontaneous AR (18 hours), $P < .05$; C, values were significantly different from those of P-induced AR (2 hours), $P < .05$; and D, values were significantly different from those of P-induced AR (18 hours), $P < .05$.

‡ Capacitated sperm were incubated first with CH (1 μg/mL, 10 minutes) and then with P_4 (10 μg/mL, 30 minutes).

§ Cholesterol (1 μg/mL, 10 minutes) was coincubated with sperm (modified HTF, 2–18 h), and P_4 (10 μg/mL, 30 minutes) was then added to induce AR.

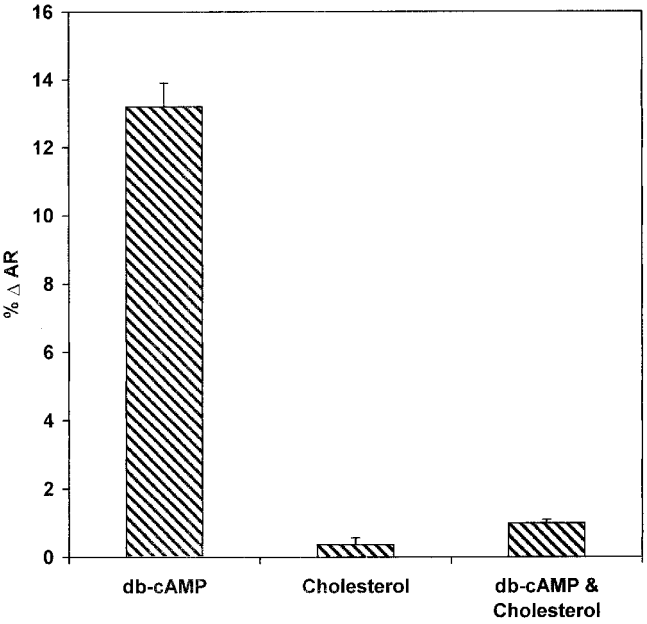

Figure 1. Inhibitory effects of cholesterol on db-cAMP–induced acrosome reaction (AR; n = 6). The percent changes in acrosome reaction (% Δ AR = $AR_{\text{treatment after 2 hours}} - AR_{\text{spontaneous AR at 2 hours}}$) were compared when db-cAMP (0.1 mM), cholesterol (1 μg/mL), or both were separately incubated with capacitated sperm in human tubal fluid.

Inhibitory Effects of Cholesterol on Spontaneous and Progesterone-Induced Acrosome Reaction

After 18 hours of incubation in capacitation medium, the number of spontaneously acrosome-reacted spermatozoa increased by as much as 5% ± 1.2%. Sperm that were co-incubated with 1 μg/mL of cholesterol resulted in a 74.6% reduction in the percentage of spontaneously acrosome-reacted sperm. Cholesterol was also observed to inhibit P_4-induced acrosome reaction following both long incubation (74.4% and 82.5% inhibition at 2 and 18 hours incubation, respectively) and short incubation (82.6% and 94.8% inhibition at 2 and 18 hours incubation, respectively). Cholesterol in concentration of 1 μg/mL was also shown to inhibit spontaneous acrosome reaction after 18 hours of incubation in capacitation medium. Results of these studies are presented in Table 1.

Effects of Cholesterol on db-cAMP-Induced Acrosome Reaction

After 2 hours of capacitation in modified HTF, a 30-minute incubation of sperm with 0.1 mM of db-cAMP resulted in an increase in acrosome reaction by 13.2% ± 0.7% ($P < .05$; Figure 1). In contrast, adding 1 μg/mL of cholesterol alone resulted in little change in acrosome reaction (0.4% ± 0.2%). Similarly, when both 1 μg/mL of cholesterol and 0.1 mM of db-cAMP were added to sperm that had been previously capacitated, the increase in the number of acrosome-reacted sperm was imperceptible (1.0% ± 0.1%) in 6 repeated experiments.

Comparison of db-cAMP- and Progesterone-Induced Acrosome Reaction

In separate experiments, the degree of acrosome reaction induction by db-cAMP was compared with that of P_4. The percent change of acrosome-reacted sperm significantly increased to 7.5% ± 1.1%, 12.3% ± 0.8%, and 15.7% ± 0.8% when 10 μg/mL of P_4, 0.1 mM of db-cAMP, and both were added to sperm, respectively (in all cases, $P < .05$; Table 2).

Table 2. *Comparison of progesterone- and db-cAMP–induced acrosome reaction (AR) (n = 6)**

	Percentage Acrosome-Reacted Sperm†
	Sperm Capacitation Time (2 hours)
Treatments	
Spontaneous AR (2-hour incubation)	2.9 ± 0.8 A
P_4 (10 μg/mL)	7.5 ± 1.1 B
db-cAMP (0.1 mM)	12.3 ± 0.8 B, C
P_4 (10 μg/mL) + db-cAMP (0.1 mM)	15.7 ± 0.8 B, C, D

* Values are reported as the mean value ± standard error of the mean.

† Values are the differences between AR at time 0 and after each treatment. A indicates values were significantly different from those of control (0 hours), $P < .05$; B, values were significantly different from those of spontaneous AR (2 hours), $P < .05$; C, values were significantly different from those of P-induced AR (2 hours), $P < .05$; and D, values were significantly different from those of db-cAMP–induced AR (2 hours), $P < .05$.

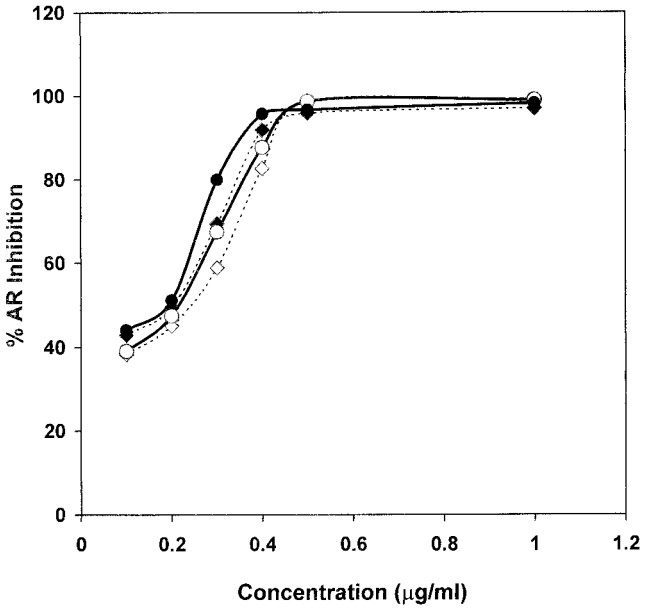

Figure 2. Dose-dependent inhibition curves of cholesterol and β-sitosterol (0–1 μg/mL) on sperm acrosome reaction induced separately by 1 and 10 μg/mL of progesterone (n = 6). – – –◊– – – indicates cholesterol + progesterone (1 μg/mL); – – –◆– – –, cholesterol + progesterone (10 μg/mL); ———○———, β-sitosterol + progesterone (1 μg/mL); ———●———, β-sitosterol + progesterone (10 μg/mL).

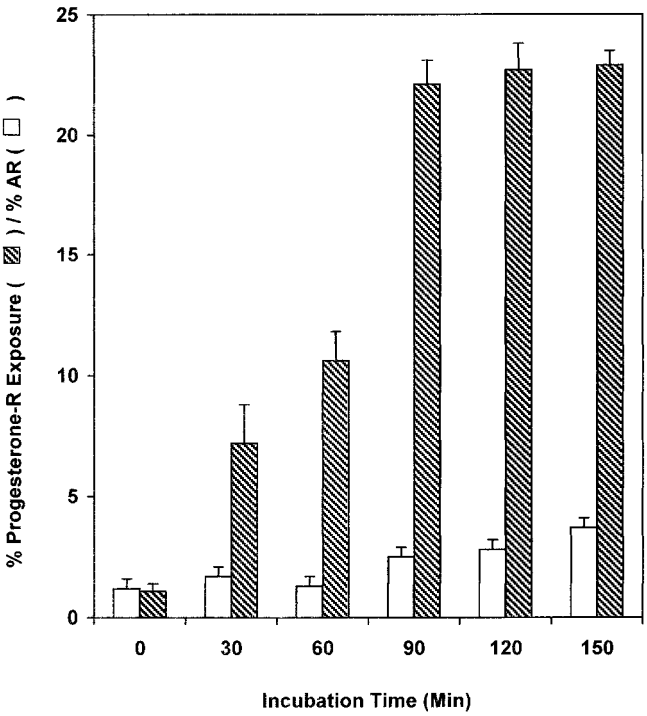

Figure 3. Percentages of human sperm progesterone surface receptors (▨) exposed as a function of time (0–150 minutes) during capacitation in modified human tubal fluid medium (n = 6). Percentages of acrosome-reacted sperm (□) as a function of incubation time in capacitation medium.

Effects of Cholesterol and β-Sitosterol on Progesterone-Induced Acrosome Reaction

The percentage of acrosome reaction inhibition did not differ significantly following treatment with cholesterol concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, or 1 μg/mL, regardless of whether 1 or 10 μg/mL of P_4 was used to induce an acrosome reaction (Figure 2). The dose-dependent inhibition curves were virtually identical for the 2 concentrations of P_4. The concentration of cholesterol that resulted in a 50% acrosome reaction inhibition was about 0.2 μg/mL; the concentration of cholesterol that would almost completely inhibit P_4-induced acrosome reaction was around 0.5 μg/mL. The statistics for dose-dependent inhibition when β-sitosterol was used were also similar to those of cholesterol and was independent of P_4 concentrations.

Time-Dependent Appearance of Progesterone Surface Receptor

We monitored the time-dependent appearance of the P_4 surface receptor by using P_4-FITC-BSA as a probe. At the beginning of treatment and incubation, less than 2% of motile human sperm showed positive staining (Figure 3), but positive fluorescent staining increased significantly with time and reached a maximum of 22.1% ± 1% after 90 mminutes of incubation. Longer incubation times of up to 240 minutes did not result in a higher percentage of positive staining. In contrast, the acrosome of the majority of sperm remained intact during the same capacitation period in 5 duplicate experiments. At the beginning of incubation in the capacitation medium, 98.8% ± 0.7% of the sperm population were acrosome-intact, whereas at 120 minutes, 97.2% ± 0.9% were acrosome-intact.

Effects of Progesterone, Cholesterol, and db-cAMP on the Appearance of Progesterone Receptors

The addition of 10 mg/mL of P_4 completely blocked acrosome staining by P_4-FITC-BSA. Likewise, little staining was observed when P_4 and cholesterol were both added. When sperm were treated with db-cAMP, the percentage of cells that showed positive staining was 28.1% ± 0.6% (Figure 4).

The addition of cholesterol alone completely inhibited the induction of acrosome reactions by db-cAMP and also prevented staining, and adding cholesterol only to the capacitation medium also completely blocked fluorescent staining. When P_4-FITC-BSA was used to stain freshly prepared sperm that were first fixed with methanol, almost all of the sperm (96.2% ± 1.8%) were stained with the fluorescein-labeled P_4 probe.

Correlation between Time-Dependent Appearance of Progesterone Receptors and Induced Acrosome Reaction

Because the appearance of the P_4 surface receptor during sperm capacitation is time-dependent (Figure 3), we chose

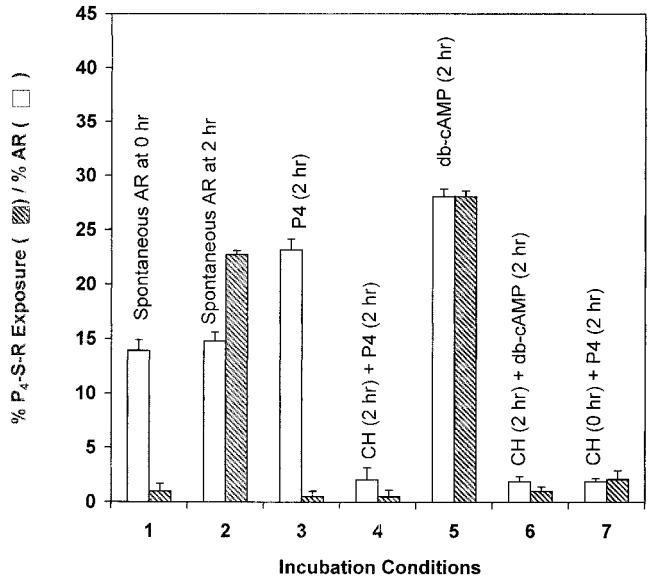

Figure 4. Effects of various inducers and cholesterol on the percentage of human sperm progesterone surface receptors exposed (▨) after 2-hour incubation in modified human tubal fluid for capacitation (n = 5). Percentage of acrosome reacted sperm (□) determined after each treatment.

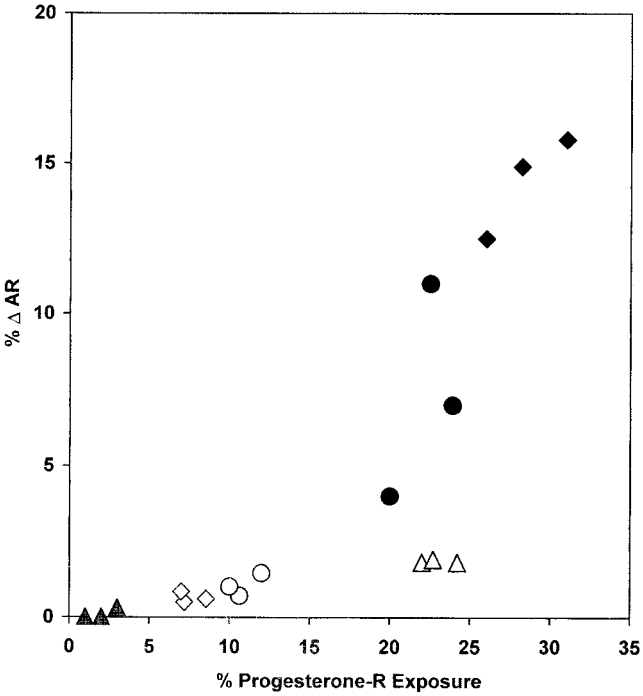

Figure 5. Relationship observed between the percentage of sperm with progesterone receptor appearance and percent changes of an induced acrosome reaction (AR; % Δ AR = $AR_{treatment\ after\ x\ hours}$ − $AR_{spontaneous\ at\ x\ hours}$). In 2 separate sets of experiments, comparisons of capacitated sperm were made for induced AR following staining with fluorescein isothiocyanate–labeled bovine serum albumin (FITC-PSA) and staining with progesterone–FITC-BSA. This comparison was made among the following different conditions (n = 3): ▲ = no incubation time (+ progesterone as an AR inducer); ◇ = 30 minutes incubation (+ progesterone as an AR inducer); ○ = 60 minutes incubation time (+ progesterone as an AR inducer); △ = 90 minutes incubation time (+ progesterone as an AR inducer); ● = 120 minutes incubation time (+ progesterone as an AR inducer); ◆ = 120 minutes incubation time (+ dibutyryl cyclic adenosine monophosphate as an AR inducer).

to study whether induction of acrosome reaction by P_4 was also a function of capacitation time and its relationship to the exposure of the P_4 surface receptor. The percentages of sperm that stained positive with P_4-FITC-BSA were 2% ± 0.6%, 7.6% ± 0.5%, 10.8% ± 0.6%, 22.9% ± 0.6%, and 22.2% ± 1.2% after 0, 30, 60, 90, and 120 minutes of incubation, respectively (Figure 5). Incubation times of more than 120 minutes did not result in any additional increase in the percentage of stained sperm. The corresponding percentages of acrosome reaction changes were 0.1% ± 0.1%, 0.5% ± 0.7%, 1.1% ± 0.2%, 1.8% ± 0.1%, and 7.3% ± 2%, respectively. Thus, the degree of acrosome reaction induced by P_4 was correlated with the appearance of the P_4 surface receptor. When db-cAMP was added after 120 minutes of capacitation, the percentage of sperm that showed P_4 receptors was 27.9% ± 0.8%, whereas the percentage of acrosome-reacted sperm was still only 13.8% ± 0.6%.

Changes were also observed in the P_4 receptor distribution on the sperm plasma membrane according to the amount of time cells remained in the capacitation medium. At the beginning of sperm capacitation, receptors were observed as fluorescent patches, distributed uniformly over the sperm head. With increasing incubation times, P_4 receptors were found mostly around the equatorial segment. After 1.5 to 2 hours in the capacitation medium, receptors were observed only at the equatorial segments or in the postacrosomal regions. Longer incubation, of up until 240 minutes, did not have any additional effect on the distribution of P_4 surface receptors. After acrosome reaction was induced by P_4 or db-cAMP, P_4 receptors remained in the equatorial region of the acrosome.

Discussion

Our findings confirm that both spontaneous acrosome reaction and those induced by P_4 were significantly inhibited by cholesterol. The cellular events that result in acrosome reaction have been demonstrated to involve at least 2 signal transduction pathways (Doherty et al, 1995), one of which involves generating a second messenger, cAMP, which ultimately activates cAMP-dependent PKA (De Jonge et al, 1991). Cholesterol has been shown to regulate acrosome response to P_4 (Cross, 1996b; 1998); however, a better understanding of cholesterol's mechanism of action remains to be elucidated. Based on our results, cholesterol's inhibition of acrosome reaction induced by P_4 is independent of P_4 concentration (Figure

2). This suggests that cholesterol inhibits the acrosome reaction in a noncompetitive manner. In our study, induction of acrosome reaction by the cAMP analogue, db-cAMP, was significantly inhibited by exogenous cholesterol (Figure 1). This indicates that db-cAMP was probably prevented from crossing the cell membrane to activate the PKA signal transduction pathway in the presence of exogenous cholesterol. It is likely that incorporation of cholesterol into the sperm plasma membrane alters the membrane's rigidity and its permeability to db-cAMP.

Similarly, β-sitosterol, a plant analogue of cholesterol, inhibited the acrosome reaction in an identical manner to that of cholesterol. Thus, it is unlikely that cholesterol exerts its action via a specific receptor-mediated pathway; instead, it is likely that cholesterol and β-sitosterol exert a common effect on the sperm plasma membrane (ie, they change the sperm plasma membrane rigidity), so that P_4 surface receptors are unable to interact with their corresponding ligands.

The presence of P_4 receptors in the human sperm plasma membrane has been reported by several previous studies (Blackmore et al, 1994; Blackmore & Lattanzio, 1991a; Aitken et al, 1996b; Alexander et al, 1996), and Revelli et al (1998) have proposed a model of nongenomic action of P_4 on reproductive tissues. However, there is an apparent contradiction among the studies on the P_4 receptor, which is probably because sperm have different types of P_4 receptors. Several researchers have demonstrated that P_4 binding to surface receptors is followed by receptor aggregation (Tesarik et al, 1992; Mendoza and Tesarik, 1993; Benoff et al, 1995; Jacob et al, 1998), stimulation of rapid Ca^{2+} influx, and acrosome reaction (Tesarik and Mendoza, 1992; Mendoza and Tesarik, 1993; Benoff et al, 1995; Jacob et al, 1998). As in previous studies, we used P_4 conjugated to BSA as a P_4 analogue. This P_4 conjugate does not enter sperm but it can initiate an acrosome reaction and an increase in Ca^{2+} concentrations (Meizel and Turner, 1991). Consistent with previous findings, only a small percentage of the sperm population was labeled by P_4-FITC-BSA (Blackmore and Lattanzio, 1991b; Tesarik et al, 1992a; Mendoza and Tesarik, 1993; Aitken et al, 1996b) in a time-dependent manner (Figure 3) during the first 2 hours of incubation in a capacitation medium; nevertheless, a majority of sperm acrosomes remained intact during the same period. When P_4 or db-cAMP was added after 2 hours of incubation, acrosome reaction induction increased proportionally with the number of exposed P_4 surface receptors. Our finding that the effects of db-cAMP on the P_4 surface receptor is inhibited by cholesterol further supports the hypothesis that cholesterol exerts its effect on the sperm plasma membrane, probably by altering the membrane's fluidity and, consequently, preventing exposure of the P_4 surface receptor.

The degree to which the acrosome reaction was induced did not appear to be related to an increase in P_4 receptor exposure during the initial capacitation period when sperm were not optimally capacitated (Figure 5). However, after 90 minutes of in vitro capacitation, acrosome reaction induction seemed to increase significantly and was correlated with receptor exposure. The degree of P_4 surface receptor exposure was much greater than the induction of acrosome reaction by P_4 or by db-cAMP (Figure 5). This observation can be explained by the findings that P_4 binding to its surface receptors can lead to 3 different signal transduction pathways, only one of which results in an acrosome reaction (Tesarik et al, 1996). Moreover, P_4 receptor exposure may serve to potentiate the acrosome reaction when a sperm cell binds to the zona pellucida (Jacob et al, 1998; Benoff, 1999).

Based on the results of our study with methanol-fixed sperm, P_4 surface receptors appear to exist in the majority of sperm; however, only a small portion of the receptors were exposed in live sperm preparation. Similar findings have been reported by other investigators (Tesarik et al, 1996). Further research is in progress to study why only a small population of spermatozoa have exposed P_4 receptors and to determine the relevance of this during fertilization.

In conclusion, the results of our study suggest that cholesterol inhibits acrosome reactions that are induced by P_4 or db-cAMP in a noncompetitive manner by modifying the surface structure of the sperm plasma membrane; in effect, by masking the P_4surface receptors. Therefore, reversible cholesterol removal from the sperm plasma membrane and the subsequent exposure of P_4 surface receptors are interrelated and important events during the process of sperm capacitation. It remains to be explained why only a minor population of sperm cells are selected to have the initial P_4-induced response.

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