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Effect of Dilauroylphosphatidylcholine on the Acrosome Reaction and Subsequent Penetration of Bull Spermatozoa into Zona-Free Hamster Eggs

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ABSTRACT

Incubation of bull sperm with liposomes made with phosphatidylcholine (PC) containing fatty acyl chains of either 10 (PC10) or 12 (PC12) carbons resulted in greater than 90% of the sperm exhibiting an acrosome reaction (AR) within 15 min. Liposomes of PC10 rapidly destroyed sperm motility while PC12 acrosome-reacted sperm remained motile for several h. Liposomes of PC with ⩾14-carbon fatty acyl chains had no effect on the AR or motility of sperm. The AR was not induced by lysophospholipids, because lysophospholipids were not detected in the PC liposomes, and the AR did not occur when lysophospholipids were tested at the same concentration as PC12. The concentration of PC12 necessary to induce maximal numbers of acrosome-reacted sperm varied with the concentration of sperm. The effect of PC12 on sperm also varied with the ratio of live to dead sperm in a sample. When 3 × 10^6 bull sperm/ml were treated with 0, 10, 20, and 30 μM PC12 for 7 min prior to addition to zona-free hamster eggs, 6, 6, 98, and 77% of the eggs were penetrated, respectively. Lipid concentrations of 0 μM and 10 μM did not affect the AR, whereas higher levels induced the AR in sperm. This procedure can quickly provide acrosome-reacted bull sperm for use with various in vitro fertilization procedures and for assessment of male fertility.

INTRODUCTION

Freshly ejaculated bovine sperm are unable to penetrate the zona pellucida of homologous eggs or the vitelline membrane of zona-free hamster eggs without first undergoing an acrosome reaction (AR). The natural stimulus for the AR is unknown. However, it can be induced artificially by several means. Follicular fluid has been reported to enhance the AR in mouse sperm (Iwamatsu and Chang, 1969). Lenz et al. (1983) reported that chondroitin sulfate, a component in follicular fluid, could induce the AR of bull spermatozoa in vitro, as evidenced by an acrosomal staining technique, electron microscopy, and the penetration of bovine oocytes.

Yanagimachi and Usui (1974) demonstrated the importance of an influx of extracellular Ca^{++} into the sperm to induce the AR. Calcium ionophores, therefore, have been used successfully to induce the AR in sperm from laboratory animals (Yanagimachi, 1975; Green, 1976, 1978; Summers et al., 1976; Talbot et al., 1976) and from the bovine (Byrd, 1981; Takahashi and Hanada, 1984).

The AR involves membrane fusion. Several compounds that either induce membrane fusion or change membrane fluidity have been used to induce the AR. The detergent Triton-X 100 induces and AR in guinea pig sperm, allowing them to penetrate zone-free hamster eggs (Yanagimachi, 1975). The addition of A_2C, an artificial membrane modifier (Fleming et al., 1982), increases membrane fluidity, as do unsaturated fatty acids, and induces the AR in guinea pig spermatozoa, enabling them to penetrate zona-free hamster eggs. The addition of lipids, such as lysophospholipids, can induce membrane fusion and the acrosome reaction in guinea pig spermatozoa (Fleming and Yanagimachi, 1981). Similarly, the addition of unsaturated free fatty acids to spermatozoa can induce the AR in hamster sperm (Meizel and Turner, 1983).

Many parameters of semen such as morphology and motility of sperm as well as metabolic or enzyme analyses have been used to predict fertility in bulls, but they have had limited success (Graham et al., 1980). The zona-free hamster eggs penetration assay has been used to test the fertility of human sperm (Overstreet et al., 1980; Hall, 1981; Zausner-Guelman

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et al., 1981; Rogers et al., 1983). Brackett et al. (1982) reported that unfrozen spermatozoa from a bull with good fertility penetrated 49% of the hamster eggs inseminated, while sperm from a bull with lower fertility penetrated 81% of inseminated hamster eggs. No correlation between egg penetration or acrosome status and bull fertility was reported when sperm from 16 bulls were treated with a high osmolality buffer (Miller et al., 1985), but no data were presented. The use of chondroitin sulfates to induce the AR in bull sperm in vitro was related to the nonreturn rates of eight dairy bulls (Ax et al., 1984). No assays of egg penetration were conducted.

The objective of this study was to evaluate specific phosphatidylcholine liposomes as a means of inducing the AR in bull sperm and testing these sperm in the assay of hamster-egg penetration.

MATERIALS AND METHODS

Experimental Design

Six experiments were conducted to investigate the effects on the AR of bull spermatozoa exerted by liposomes made from synthetic phosphatidylcholines (PC). In the first experiment, sperm from four bulls were treated with liposomes made from either dicaproyl-PC (PC10), dilauroyl-PC (PC12), dimyristoyl-PC (PC14), dipalmitoyl-PC (PC16), distearoyl-PC (PC18), or diarachidoyl-PC (PC20). Sperm samples were assayed 15, 30, and 60 min after liposomes were added for the percentage of motile sperm and for the percentage of sperm exhibiting an AR.

In a second experiment, sperm from seven bulls were treated with five concentrations of PC12 liposomes. After liposomes were added, the sperm samples were assayed at 15, 30, 60, 90, 120, and 180 min for the percentage of motile sperm and the percentage of sperm showing an AR.

A third experiment was conducted to determine more fully the nature of the liposomes that induce the AR in bull sperm. This was done by using thin-layer chromatography (TLC) to identify any lipids besides the PC12 liposomes. Liposomes made from either PC12 or from lysolauroyl-PC (lysoPC12) (a possible breakdown product of PC12) were also incubated with sperm to determine if lysoPC12 was inducing the AR in bull sperm.

The fourth experiment was conducted to determine if the concentration of sperm cells affected the AR induced by PC12 liposomes. Three concentrations of PC12 liposomes and three concentrations of sperm were included in a factorial arrangement.

It had been casually observed that in ejaculates with more dead sperm initially, the motile sperm unexpectedly survived longer, so a fifth experiment was conducted to test specifically the effects when the ratio of live to dead sperm cells varied. This ratio was changed by taking one aliquot of cells killed by plunging into liquid nitrogen and combining them with live cells so that dead cells added represented 100, 75, 50, 25, and 0% of the mixture. In addition, ^{14}C-labeled PC12 was added to sperm samples containing washed cells, which were either alive or killed, to determine if lipid uptake by cells depended upon their viability.

Finally, the sixth experiment was conducted to determine if bull sperm exhibiting an AR induced by PC12 liposomes were capable of penetrating zona-free hamster eggs.

Preparation of Liposomes

Liposomes were prepared from the desired phospholipids obtained >99% pure in chloroform from Avanti Polar Lipids Inc., Birmingham, AL. In the first experiment, phospholipids containing fatty acyl chains of different length were compared. Approximately 8.7 μmol of lipid (4.9, 5.4, 5.9, 6.4, 6.9, and 7.3 mg of PC10, PC12, PC14, PC16, PC18, and PC20, respectively) were added. In the experiments involving lysoPC12 and PC12, 3.8 mg of lysoPC12 or 5.4 mg of PC12 were added to tubes, also giving approximately 8.7 μm of either lysoPC12 or PC12. Each tube was flushed with nitrogen gas until all the solvent was dryed. The tubes were then purged an additional 5 min. Control tubes in which chloroform but no lipids were added were prepared in the same manner. A Ca^{++}-free Tyrodes medium (NaCl; 94.3 mM; KCl, 4.0 mM; Na_2HPO_4, 0.7 mM; $MgCl_2 \cdot 6H_2O$, 0.5 mM; $NaHCO_3$, 25.9 mM; N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), 20.0 mM; glucose, 12.8 mM; Na pyruvate, 1.3 mM; lactate, 7.6 mM and bovine serum albumin (BSA), 3 mg/ml), which will be referred to as Ca^{++}-free TALP, was adjusted to a pH of 7.45 and an osmolarity of 300 mOsm. A volume of 12 ml of this medium was added to each tube, placed in a 37°C water bath, and sonicated for 5 min with a Branson Sonifier (Branson Instruments Co., Stamford, CT) at an output setting of 2. Each tube was continuously purged with nitrogen gas during sonication. After sonication each

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solution was centrifuged at 27,000 × *g* for 15 min to separate any undispersed lipid and any titanium lost from the tip of the sonifier. Only trace amounts of lipid precipitated. The supernatant was decanted into a glass tube and purged with nitrogen gas. Aliquots of 0.5 ml of the lipid suspension were placed in cryovials (Wheaton Scientific, Millville, NJ), attached to metal freezing canes, and plunged into liquid nitrogen. The liposomes were stored under nitrogen at −196°C until used.

Frozen liposome samples were used immediately after thawing at room temperature. A single lipid batch was used in each experiment. The lipid concentration of the liposomes was determined by using ammonium ferrothiocyanate as described by Stewart (1980).

The ^{14}C-radiolabeled PC12 was prepared according to the procedure of Aneja and Chadha (1971). Liposomes were prepared in Ca^{+}-free TALP, as described by Crain and Zilversmit (1980), and were used immediately after preparation.

Lipids were extracted from the liposomes for TLC analysis by the Folch extraction method described by Radin (1969). Approximately 25 μl of lipid standards (PC12 and lysoPC12, > 99% pure) at 1 mg /ml and 50 μl of lipid extracted from the liposomes were applied 3 cm from the edge of a 20 × 20 cm Supelco TLC plate (Supelco, Inc., Bellefonte, PA). These plates were covered 0.25-mm thick with silica gel-H with SiO_2 as a binder. The TLC plates were developed with a mixture of chloroform, methanol, acetic acid, and water 25:15:4:2 (v/v). The lipids were detected by spraying the plates with 50% sulfuric acid and then heating the plates in an oven at 180°C for 30 to 60 min, until the lipid spots turned brown. The distance each lipid spot had moved from the origin was measured, and the retardation-factor (Rf) value for each lipid spot was determined.

Collection of Zona-free Hamster Eggs

Zona-free eggs were obtained by the procedure described by Yanagimachi et al. (1976), except that a different medium was used. Oviducts were removed from female hamsters previously superovulated with 25 IU pregnant mare's serum (PMS) followed 49 to 53 h later by 20 IU human chorionic gonadotropin (HCG). The oviducts were washed in TALP (NaCl, 97.4 mM; KCl, 3.1 mM; Na_2HPO_4, 0.3 mM; $NaHCO_3$, 24.9 mM; $CaCl_2 \cdot 2H_2O$, 0.4 mM: HEPES, 10 mM; Na-Pyruvate, 1.2 mM; lactate, 13.1 mM; glucose, 5 mM and BSA, 6 mg/ml) at 35°C. The buffered TALP had a pH of 7.4. The cumulus mass was removed from the oviduct and the cumulus cells dispersed with 0.1% hyaluronidase. The zona pellucidae were removed with 0.1% trypsin. The eggs were washed three times in fresh TALP at 25°C, and groups of four eggs were placed with 30 μl TALP in a plastic petri dish (Falcon, Oxnard, CA) and covered with 4 ml Dow Corning 360 Medical Fluid, viscosity 20 centistokes (Dow Corning Corp., Midland, MI). The eggs were then incubated at 39°C under an atmosphere of 5% CO_2 in air for 10–20 min until they were inseminated with acrosome-reacted sperm.

Preparation of Sperm

Ejaculates of bull semen were obtained from Eastern A.I. Cooperative, Ithaca, NY, and brought to the lab within 1 h. The sperm cells were washed by diluting 1 to 2 ml of semen to 11 ml with 10 mM Ca^{++}-TALP (same composition as TALP, except for 10 mM $CaCl_2 \cdot 2H_2O$ and 92.9 mM NaCl). The cells were centrifuged twice at 650 × *g* for 5 min, the supernatant removed by aspiration, and the sperm pellet resuspended to 11 ml after the first wash and to approximately 1 ml after the second wash with 10 mM Ca^{++} TALP. The concentration of the washed sperm was determined with a Coulter Counter (Model ZB_1, Coulter Electronics, Hialeah, FL). The sperm were diluted to 150×10^6, 100×10^6, and 50×10^6 sperm/ml. In the experiment on penetration of hamster eggs, the sperm were diluted to 6×10^6 sperm/ml.

Sperm treated with radiolabeled liposomes were diluted to 100×10^6 sperm/ml and the sample divided. One half of the sample was killed by plunging it into liquid nitrogen for 5 min before thawing. The other half of the sample was maintained at 25°C.

Washed sperm were treated by adding 200 μl of Ca^{++}-Free TALP, in which liposomes (made in Ca^{++}-free TALP) had been mixed at twice the desired lipid concentration to 200 μl of the washed cells. This mixture was incubated in a water bath at 39°C. At the indicated times, 6-μl samples were removed for estimates of sperm motility and for determination of AR. Motility estimates were made by a single observer using bright-field microscopy with a 10× objective connected to a television monitor (700× total magnification). This permitted visualization of individual sperm cells. Sperm were considered motile if they

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swam progressively forward, or, if stuck to the slide, showed vigorous flagellar movement.

Slides for the estimates of AR were made by spreading 6 μl of sample over a single slide heated to 35°C. Slides were dried for 24 h at 25°C before being stained by the procedure of Lenz et al. (1983). These slides were examined with phase microscopy and 100 sperm/slide observed for the AR. Sperm were stained with an eosin-aniline blue stain mixture (Shaffer and Almguist, 1948) to estimate the percentage of live sperm in the second experiment.

Sperm to be added to hamster eggs were incubated with the liposomes for 7 min at 39°C. Then 30 μl aliquots (100,000 sperm) were added to replicates of four hamster eggs in 30 μl TALP.

Incubation of Sperm and Eggs

The eggs were inseminated with liposome-treated sperm and incubated at 39°C for 3 h under an atmosphere of 5% CO_2 in air. After incubation, the eggs were washed once in TALP to remove excess sperm and placed on a glass slide with 9–11 μl TALP. A coverslip having a spot of paraffin/vaseline mixture (1:10) on each corner was then placed over the eggs and pressed until the eggs were held securely in place, but not ruptured. Drops of rubber cement were placed along two edges of the coverslip to secure it to the slide. The eggs were then fixed by immersing the slide in alcohol: acetic acid (3:1) overnight.

After fixation, the eggs were stained with 1% lacmoid in 45% glatial acetic acid and observed under phase microscopy at 500X for sperm penetration. An egg was considered penetrated when at least one swollen sperm head (or sperm pronucleus) and its associated tail were visible within the egg (Fig. 1).

Radiolabeled Sperm

Sperm were labeled by placing 200 μl of sperm, 200 μl of liposomes (100 nM ^{14}C-labeled PC12), and 100 μl TALP into 1.5 ml-capacity microcentrifuge tubes (West Coast Scientific, Inc., Emeryville, CA). After incubations of 0, 5, 15, 30, and 60 min, 50 μl 0.5 M NaN_3 was added to stop lipid transfer, and the samples were centrifuged for 2 min to pellet the sperm cells. A 300-μl volume of the supernatant was removed and the radioactivity measured with a Beckman LS-250 liquid scintillation counter (Beckman Instr., Palo Alto, CA). The percentage of PC12 transferred to the sperm cells was determined as described by Crain and Zilversmit (1980).

Statistical Analysis

Data for Experiments 1 and 6 were analyzed by analysis of variance (SAS Inst. Inc., 1982) and the treatment means tested by a set of orthogonal contrasts. The analysis of variance was calculated by the model: $Y_{ijk} = u + B_i + T_j + BxT + E_{ijk}$, where B = i^{th} bull, T = j^{th} treatment, B×T = interaction between bulls and treatments, and E_{ijk} = residual. Analyses were conducted on the arcsin transformation of the percentages of motile cells, sperm exhibiting an AR, and eggs penetrated by sperm. Bulls were considered as a random variable and treatments as fixed. Bulls generally differed with respect to motile spermatozoa and AR, and it was important to isolate this variance in the model. However, the statistical significance of this variable was of minor interest in these studies and is largely ignored in discussion of results.

For Experiments 2 through 5, multivariate analysis for repeated measures was used for both variables measured. When the percentage of motile sperm was measured, the decline in motility over time was first

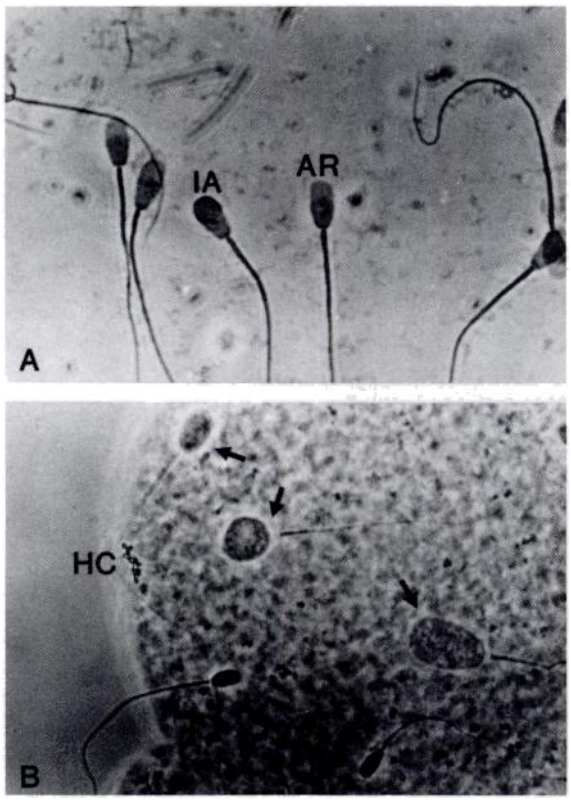

FIG. 1. (A) Bull spermatozoa fixed and stained to reveal cells with intact acrosomes (IA) and cells exhibiting an acrosome reaction (AR). ×750. (B) Zona-free hamster egg, penetrated with bull sperm, undergoing nuclear decondensation (*arrows*). Two other sperm appear on the oocyte surface. Also, hamster chromosomes (HC) are visible. ×600.

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determined. These slopes were then analyzed by analysis of variance using a general-linear-models procedure (SAS Inst. Inc., 1982) and the treatment means tested by using a set of orthogonal contrasts. The percentage of sperm exhibiting an AR appeared to follow a negative exponential curve ($AR=B_0[1-e^{-B[T]}]$) where T is time in min. Therefore, nonlinear regression procedures using Marguardt's method (SAS Inst., Inc., 1982) were used to determine the maximum percentage of AR sperm in each treatment. The data were then analyzed by analysis of variance, using a general-linear-models procedure, and the treatment means were tested by orthogonal contrasts.

RESULTS

Experiment 1

The percentage of motile sperm and of sperm exhibiting an AR after incubation with 70 μM PC in which the PCs contained fatty acyl chains of 10 to 20 carbons are presented in Table 1. Sperm treated with PCs having ⩾14 carbons in the fatty acyl chains had less than 4% cells exhibiting an AR. After a 15-min incubation with liposomes, >90% of the sperm in samples treated with PC10 or PC12 exhibited an AR. Sperm treated with PC10 had no motility after 15 min, but sperm treated with PC12 maintained a high percentage of motile cells at 30 min. Nearly all the sperm treated with PC12 stuck to the glass slide in the head region while their tails beat vigorously. The few sperm not stuck to the slide often exhibited a whiplash or figure-8 type of motility typical of hyperactivated sperm (Yanagimachi, 1970).

Experiment 2

The percentage of sperm exhibiting an AR and the percentage of sperm exhibiting motility after exposure to five concentrations of PC12 liposomes are presented in Figure 2. Increasing the concentration of PC12 liposomes while maintaining a constant sperm concentration resulted in increased numbers of sperm cells that exhibited an AR as well as an increased rate of sperm death (immotile sperm). Sperm samples treated with 0, 35, 56, 77, and 98 μM PC12 produced maximum percentages of acrosome-reacted cells of 3.3, 9.7, 47.6, 71.9, and 86.1 (standard error from ANOVA = 6.9), respectively, while the linear decline in percentages of motile cell/min were 0.1, 0.2, 0.3, 0.6, and 0.9 (standard error from ANOVA = 0.1), respectively. Treatment of sperm with 35 μM PC12 resulted in percentages of AR and of motile cells that were not different from those of control samples ($p>0.05$). However, all other concentrations of PC12 resulted in percentages of AR and rates of declining motility that differed from each other ($p<0.05$).

In a subexperiment, sperm from two bulls were treated with PC12 liposomes or left untreated in the presence or absence of Ca^{++}. The percentage of sperm exhibiting an AR after 15 min incubation with liposomes was 3.0 ± 1.0 and 94.0 ± 1.0 in the absence and presence of 5 mM Ca^{++}, respectively ($p<0.05$). Without lipid, the AR was 2.5 ± 1.5% regardless of the presence or absence of Ca^{++}. Sperm motilities for these four treatments were all approximately 80% ($p<0.05$). Similar results were obtained when samples were incubated for 30 and 60 min.

When exposed to supravital stain, all liposome-treated sperm stained as if the cells were dead even

TABLE 1. Experiment I. The effect of treating semen from four bulls with vesicles of phosphatidycholine (PC) containing fatty acyl chains with different numbers of carbons.

Fatty acyl chain length (No. of carbons)	Time of reaction					
	15 min		30 min		60 min	
	Motility (%)	Acrosomes reacted (%)	Motility (%)	Acrosomes reacted (%)	Motility (%)	Acrosomes reacted (%)
No lipid	82.5^{a}	3.5^{a}	82.5^{a}	4.0^{a}	82.5^{a}	1.5^{a}
PC10	1.2^{b}	96.7^{b}	0.0^{c}	96.5^{b}	0.0^{b}	98.5^{b}
PC12	88.7^{a}	91.2^{b}	66.2^{b}	99.0^{b}	0.0^{b}	99.2^{b}
PC14	85.0^{a}	2.2^{a}	83.7^{a}	3.0^{a}	80.0^{a}	4.0^{a}
PC16	85.0^{a}	3.0^{a}	87.5^{a}	1.5^{a}	77.5^{a}	2.0^{a}
PC18	83.7^{a}	2.0^{a}	82.5^{a}	2.5^{a}	75.0^{a}	4.5^{a}
PC20	86.2^{a}	0.7^{a}	86.2^{a}	1.5^{a}	80.0^{a}	2.7^{a}
SEM	1.8	2.0	3.0	1.0	1.9	1.0

^{a,b,c} Superscripts denote differences within columns at $p<.05$

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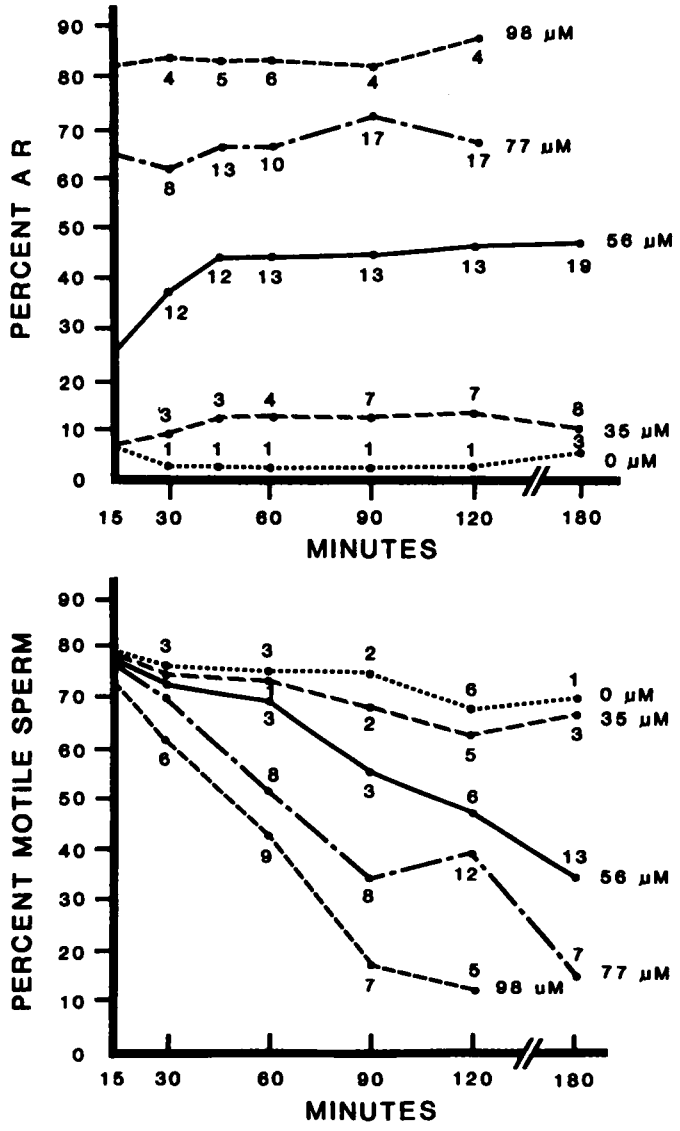

FIG. 2. Experiment 2. The percentage of acrosome-reacted (AR) sperm and the percentage of motile sperm after spermatozoa from seven bulls were treated with several concentrations of PC12 liposomes. Numbers associated with means are the SEM. At 15 min, SEM (not shown) were very small.

though they exhibited vigorous motility. Thus, the technique could not be used as an indicator of immotile dead cells.

Experiment 3

Results from TLC (Fig. 3) indicate that liposomes contained only PC12 lipids and that no lysoPC12 was formed during liposome preparation. Samples of PC12 and lysoPC12 standards had Rf values of 0.3 and 0.1 respectively. Lipids from three batches of PC12 liposomes gave spots with an average Rf value of 0.3. No spots could be detected in the area expected if lysoPC12 was present in any batch. Sonicated media without lipids resulted in no detectable spot.

As a further functional test for the presence of lysoPC12, liposomes made with PC12 and lysoPC12 at two different lipid concentrations were added to bull sperm. The percentage of motile sperm and the percentage of sperm exhibiting AR are presented in Figure 4. At both the 56- and 97-μM lipid concentrations, PC12 induced the AR ($p<0.05$), but the lysoPC12 did not affect the AR. The rate at which the percentage of motile cells declined was low for controls and for sperm treated with 56 or 97 μM lysoPC12, but was higher for AR sperm treated with 56 or 97 μM PC12 ($p<0.05$).

Experiment 4

The maximum calculated percentage of sperm exhibiting an AR (see statistical methods section) and the percentage of motile sperm in a sample when three concentrations of sperm were incubated with three levels of lipid are presented in Figure 5 and Table 2. Few AR sperm were seen at any sperm concentration when incubated without PC12. When sperm were incubated with 40 μM of PC12, samples

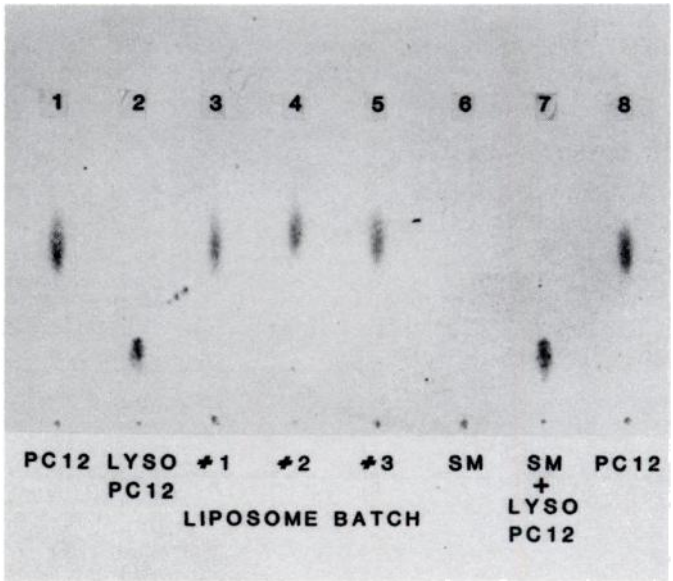

FIG. 3. Experiment 3. Thin-layer chromatogram of PC12 standard, lysoPC12 standard, and lipids extracted from PC12 liposomes. Columns: (1) PC12 standard, 25 μg; (2) lysoPC12 standard, 25 μg; (3,4,5) lipids extracted from batches 1, 2 and 3 of PC12 liposomes, 50 μl; (6) sonicated TALP medium (SM), 50 μl; (7) sonicated TALP medium + lysoPC12, 50 μl; (8) PC12 standard, 25 μg.

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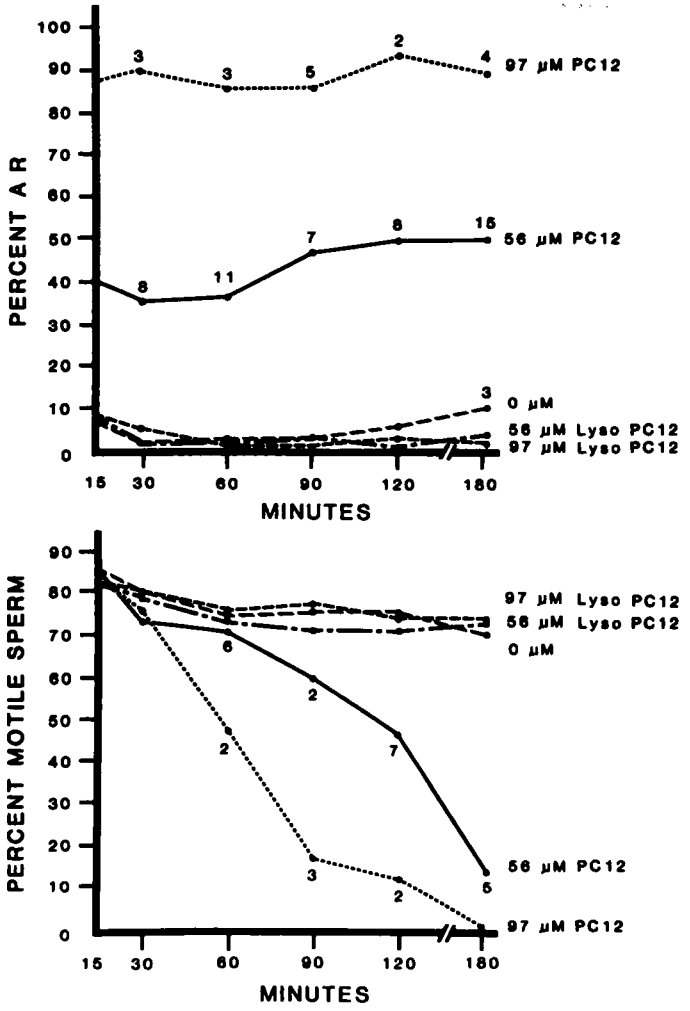

FIG. 4. Experiment 3. The percentage of acrosome-reacted (AR) sperm and the percentage of motile sperm after spermatozoa from four bulls were treated with two concentrations of PC12 liposomes, two concentrations of lysoPC12 liposomes or no lipid. Where space permitted, numbers associated with the means are the SEM. Other SEM for lysoPC12 were ≤2 for AR and ≤5 for percentage of motile sperm.

containing low numbers of sperm exhibited a higher percentage of AR sperm ($p<0.05$) than samples with high numbers of sperm. With 80 μM of PC12, all sperm exhibited a high percentage of AR sperm.

A significant interaction of lipid concentration × sperm concentration was observed for the percentage of sperm exhibiting an AR ($p<0.01$), which was due to the decline in the number of sperm undergoing an AR when sperm concentration was increased for samples treated with 40 μM PC12 (Table 2). A significant interaction of lipid concentration × sperm concentration was also observed for the rate at which the percentage of motile sperm cells declined ($p<0.01$). This interaction, however, involved all lipid concentrations and all sperm concentrations. Differences between motility of sperm cells and percentages of AR were detected for ejaculates from different bulls ($p<0.05$).

Experiment 5

The addition of 80 μM PC12 to sperm at 100 × 10^6 cells/ml, in which the number of live cells varied from 0 to 80%, resulted in approximately 90% of the sperm exhibiting an AR (Fig. 6). The rate at which the percentage of motile sperm declined was greatest with sperm samples that had the highest initial motility, and this rate decreased at each subsequent live/dead ratio ($p<0.05$).

Differences between the sperm from different bulls were detected in the percentage of motile sperm ($p<0.05$), but not in the percentage of sperm exhibit-

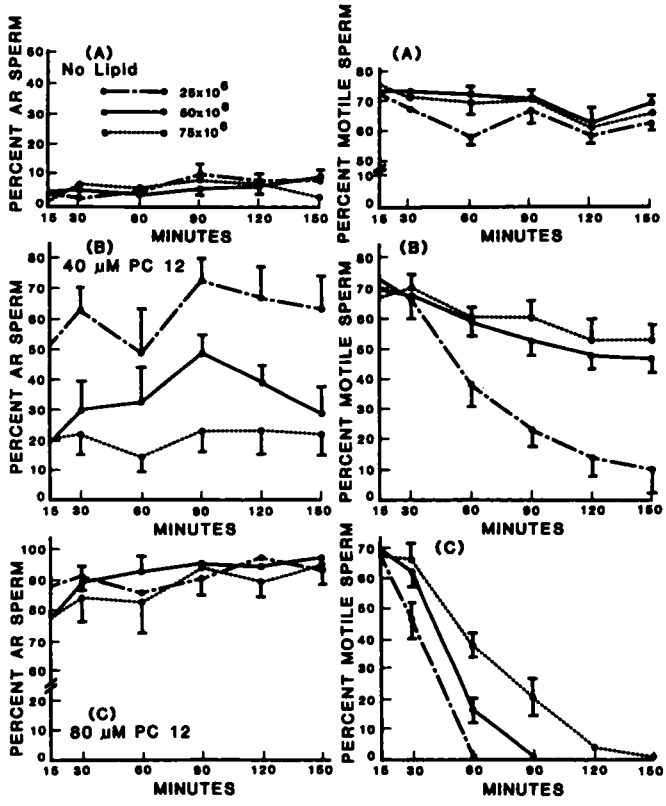

FIG. 5. Experiment 4. The percentages of acrosome-reacted (AR) sperm and the percentage of motile sperm after spermatozoa from six bulls were diluted to 25 × 10^6, 50 × 10^6, or 75 × 10^6 sperm/ml and treated with (A) no lipid, (B) 40 μm PC12 liposomes, or (C) 80 μM PC12 liposomes. *Vertical bars* are SEM, but are omitted where they were too small to illustrate or overlapped adjacent lines.

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TABLE 2. Experiment 4. Average percentage of acrosme-reacted (AR) sperm and the average deline in the percentage of motile cells/min for sperm from six bulls diluted to three different concentrations and incubated with three levels of PC12 liposomes for 150 min.

PC12 liposomes (μM)	Sperm concentration (10^6 sperm/ml) 25 Max. % AR	25 Decline in % motile sperm/min	50 Max. % AR	50 Decline in % motile sperm/min	75 Max. % AR	75 Decline in % motile sperm/min
0	8.0^{a}	0.1^{a}	4.3^{a}	0.1^{a}	6.1^{a}	0.1^{a}
40	65.0^{b}	0.5^{b}	41.7^{b*}	0.2^{a*}	24.1^{b**}	0.1^{a*}
80	90.6^{c}	1.5^{c}	92.2^{c}	1.0^{b*}	92.1^{c}	0.6^{b**}
SEM	5.0	0.1	2.8	0.1	2.7	0.04

^{a,b,c}Superscripts deonte column differences at $p<.05$.
*,**Superscripts denote row differences between like measurements at $p<.05$.

ing an AR. A bull × treatment interaction was also apparent for both the percentages of motile sperm and the AR ($p<0.05$).

The uptake of ^{14}C-PC12 by controls and by sperm killed by plunging into liquid nitrogen is presented in Table 3. Killed sperm accumulated approximately twice the amount of PC12 as did live sperm. This lipid transfer was nearly completed by 5 min.

Experiment 6

Sperm from three ejaculates from the same bull exhibited approximately 88% motile cells when incubated at 6 × 10^6 cells/ml with 0, 10, 20, or 30 μM PC12 for 15 min (Table 4). The percentage of AR sperm generally increased with increasing concentrations of lipids. Trends were similar after 30- and 60-min incubations except that the motility de-

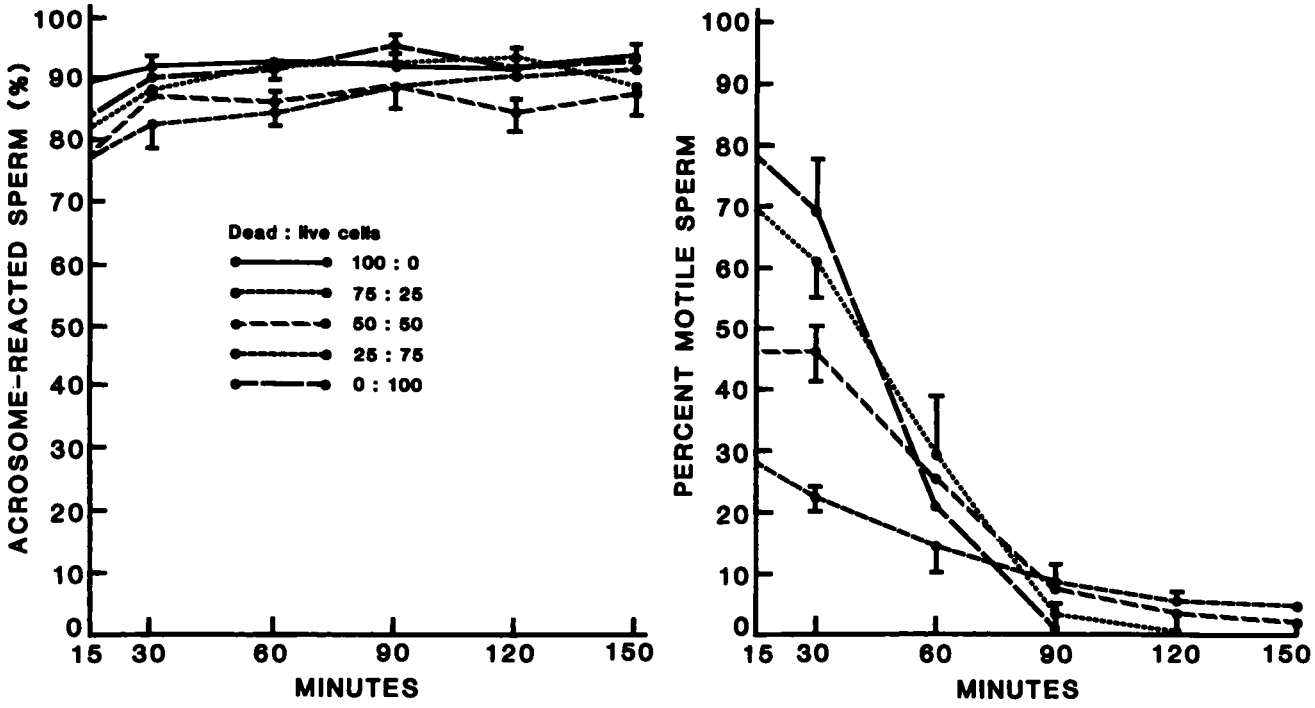

FIG. 6. Experiment 5. The percentage of acrosome-reacted sperm and the percentage of motile sperm after spermatozoa from seven bulls were diluted to 100 × 10^6 cells/ml, the samples split and half of the sperm killed by plunging them into liquid nitrogen. Dead sperm were added back to the live portion at ratios indicated. Samples were then incubated with 80 μM PC12 liposomes. *Vertical bars* are SEM.

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TABLE 3. Experiment 5. Percentage of ^{14}C-labeled PC12 transferred from liposomes to 50 × 10^6 sperm after incubation for up to 60 min when sperm were either live or dead.

Incubation time (min)	% ^{14}C-PC12 transferred to live sperm	% ^{14}C-PC12 transferred to killed sperm
0	4.8	1.6
5	24.7	51.0
15	28.7	48.1
30	33.8	61.2
60	35.5	60.7

clined more rapidly at the higher concentrations of PC12.

Samples of sperm from the same tubes incubated 3 h with eggs after a 7-min incubation with either 0 or 10 μM PC12 penetrated few of the hamster eggs (Table 5). Samples treated with 20 μM lipid penetrated more eggs, while incubation with 30 μM PC12 decreased the rate of penetration ($p<0.05$). A similar trend was observed in the total number of sperm in each penetrated egg.

DISCUSSION

Liposomes, Acrosome Reaction, and Sperm Motility

Phosphatidylcholine phospholipids having fatty acyl chains of 12 carbons or less (Table 1) were effective in inducing sperm to undergo an AR. Phosphatidylcholine containing fatty acyl chains of 14 carbons or more had no effect on sperm AR. Consistent with these results, Mashino et al. (1983) reported that the rate of PC10 transfer into human erythrocytes was greater than the rate of PC12, while PC14 showed no appreciable transfer. Similarly, PC10 liposomes caused maximal hemolysis of the erythrocytes at a lower concentration than did PC12, while PC14 liposomes failed to cause erythrocyte lysis (Mashino et al., 1983).

Nichols and Pagano (1981) and Pagano et al. (1981) also reported that fluorescently labeled PC12 was able to transfer from donor liposomes to recipient liposomes as a free monomer. Transfer equilibration between liposomes was achieved in msec (Nichols and Pagano, 1981).

Fleming and Yanagimachi (1981) reported that the addition of up to 800 μg PC/ml failed to induce the AR in guinea pig spermatozoa. They did not report what species of PC was used, but it probably was a mixture of PC species obtained from natural sources, of which PC12 is only a minor component. Other lipids mask the effect of PC12 in such preparations. The transfer of PC12 mixed 1:1 with egg PC was nearly 10 times less than the transfer from liposomes containing only PC12 (Mashino et al., 1983).

Because PC10 destroyed sperm motility very rapidly (Table 1) only PC12 liposomes were used to induce the AR in bull sperm. As the PC12 concentration was increased, the number of sperm exhibiting an AR increased in a dose-response fashion, and the percentage of motile cells declined (Tables 2, 4). These results are similar to the results on AR and sperm motility reported when hamster sperm were treated with increasing concentrations of ionophore A23187 (Talbot et al., 1976; Green, 1978).

Bull sperm treated with PC12 stuck to the glass slides as did ram sperm capacitated in vivo (Cummins, 1982). Ram sperm stuck to the slide at a site near the equatorial segment of the head, while the tail exhibited whiplash movement. Other sperm cells from the samples treated with PC12 moved in a figure-8

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TABLE 4. Experiment 6. Percentage of motile sperm and percentage of acrosome-reacted (AR) sperm when semen from three ejaculates from the same bull were treated with several levels of PC12 liposomes.

Concentration of PC12 liposomes (μM)	Time					
	15 min		30 min		60 min	
	Motility (%)	AR (%)	Motility (%)	AR (%)	Motility (%)	AR (%)
0	81.7^a	6.0^a	81.7^a	3.0^a	80.0^a	8.0^a
10	83.3^a	4.0^a	78.3^a	8.7^a	71.7^a	9.3^a
20	86.7^a	41.7^b	78.3^a	67.3^b	15.0^b	64.3^b
30	80.0^a	83.3^c	63.3^a	92.3^c	0.0^c	95.0^c
SEM	2.5	2.9	8.5	7.5	5.3	5.8

^{a,b,c}Superscripts denote differences in columns at $p<.05$.

TABLE 5. Experiment 6. Penetration rate and number of sperm penetrating 64 zona-free hamster eggs/treatment after sperm from thre ejaculates from the same bull were treated with several levels of PC12 liposomes.

Liposome concentration (μM)	Eggs penetrated (%)	No. of penetrated sperm	No. sperm/ penetrated egg
0	6^{a}	5^{a}	1.2^{a}
10	6^{a}	5^{a}	1.2^{a}
20	98^{b}	207^{b}	3.3^{b}
30	77^{c}	143^{c}	2.9^{b}
SEM	6	5	0.3

^{a,b,c}Superscripts denote differences in columns at $p<.05$.

fashion similar to those exhibiting hyperactivated motility (Yanagimachi, 1970; Fraser, 1977; Cooper et al., 1979).

The sperm treated with liposomes stained with a supravital stain, indicative of dead cells, even though the cells maintained motility. Therefore, this assay was omitted from subequent experiments. Tanaka et al. (1983) proposed that PC12 induces hemolysis in blood cells by forming "pores" in the cell membrane. If this is so, it can be hypothesized that the stain entered viable sperm cells through these pores. It may also be possible for the stain to penetrate the inner acrosomal membrane after sperm have undergone an AR.

Lysophospholipids have been implicated in the induction of the AR in guinea pig spermatotozoa (Fleming and Yanagimachi, 1981). We failed to observe any difference from control samples in the percentage of sperm exhibiting an AR when bull sperm were treated with two levels of lysoPC12 (Fig. 4). Fleming and Yanagimachi (1981) obtained 30% AR in guinea pig sperm when 8×10^6 sperm/ml were treated with 85 μg/ml of lysoPC (chain composition not specified), but only 10% AR occurred with 35 μg/ml of lysoPC. The 97-μM concentration of lysoPC12 used in the current experiment (equivalent to 42 μg/ml) incubated with 50×10^6 sperm/ml was ineffective in inducing AR, but PC12 liposomes at this concentration were very effective.

When sperm were incubated without exposure to lipids, the sperm concentration did not affect the percentages of either acrosome-reacted or motile spermatozoa. When 40 μM PC12 was added to 25×10^6, 50×10^6 and 75×10^6 sperm/ml, differences ($p<.05$) were found (Fig. 5). The AR paralleled the ratio of lipid concentration to the number of sperm cells. It is probable that at the low concentrations of sperm, sperm cells incorporate more lipid per cell because of availability of lipids than at higher concentrations of sperm. With 75×10^6 sperm/ml, only 20% of the sperm exhibited an AR, indicating either that 20% of the sperm incorporated more of the lipid or that 20% of the sperm were more susceptible to induction of the AR. At 80 μM PC12, most bull sperm exhibited an AR. These results are similar to data obtained using ionophore A23187. Increasing the concentration of sperm while the concentration of the ionophore remained the same delayed the onset of the AR in guinea pig sperm and also decreased the number of sperm undergoing the AR (Talbot et al., 1976).

Large differences in the percentage of motile sperm occurred when lipid-to-sperm concentrations were varied (Fig. 5). If this was due to more lipid incorporated with high lipid-to-sperm ratios, a faster deterioration of the acrosomal membrane, plasma membrane, and possibly the mitochondrial membranes may have occurred, thereby leading to more rapid cell death. The differences observed in the effect of PC12 upon the AR and upon sperm motility suggest that the acrosomal membrane is more sensitive to PC12 than are other sperm membranes.

When PC12 liposomes were added to sperm at a single concentration but the live/dead sperm cell ratio was varied (Fig. 6), all samples exhibited a high proportion of acrosome-reacted sperm. Unfortunately, dead sperm undergo postmortem changes and lose the main body of their acrosomes (Franklin et al., 1970). Therefore, staining for the AR does not distinguish live from dead cells. The rate at which the percentage of motile cells declined, however, could be distinguished and was different for each treatment. Samples that initially contained more live cells lost motility faster than samples that initially contained more dead spermatozoa. These dead cells accumulated more PC12 than did live cells (Table 3), leaving the live sperm cells, in treatments with many addded dead sperm, in a solution with a lower lipid concentration. This result is consistent with the experiment (Fig. 2) in which it was shown that exposure to high concentrations of PC12 not only induced an AR, but also reduced sperm motility.

Hamster-Egg Penetration

The occurrence of penetrated zona-free eggs is believed to reflect the occurrence of an AR in at least some of the viable spermatozoa (Bedford, 1983).

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Results from the final experiment (Table 5) demonstrated that sperm treated with PC12 liposomes penetrate zone-free hamster eggs. This indicated that PC12 treatment does induce a functional AR and not merely an AR associated with spermatozoan degeneration. The penetration results indicate that there was a minimum threshold in the lipid concentration required. When 1.2×10^6 cells were treated with 10 μM PC12, this threshold was not reached, few cells exhibited an AR, and few eggs were penetrated. When 20 μM PC12 was added, this threshold was surpassed. Sperm underwent an AR, and egg penetration occurred. Increasing the lipid concentration to 30 μM induced a higher percentage of acrosome-reacted cells, but sperm motility was completely eradicated by 60 min. The decrease in the rate of penetration and in the total number of sperm penetrating eggs indicate that at this concentration, the lipid had a toxic effect and sperm viability declined before many of the sperm were able to penetrate the eggs.

Mechanism of PC12 Action

The mechanism by which PC12 induced the AR in bull spermatozoa has yet to be determined. It is likely, however, that it is similar to the mechanism by which this lipid causes hemolysis in erythrocytes. Mashino et al. (1983) reported that the accumulation of PC12 by erythrocytes did not occur by fusion or by absorption between the cells and the PC12 liposomes, but that the lipid was transferred spontaneously by "free monomer diffusion" and that no transfer protein was involved. Their results also indicate that no membrane protein on the erythrocyte acts as a binding receptor for PC12, but that the binding site appears to be in the lipid bilayer of the membrane. Further studies by Tanaka et al. (1983) indicated that PC12 created "pores" in the membrane that induced membrane permeability to ions such as Na^+ and K^+ as well as glucose, but dextran and hemoglobin were unable to permeate the membranes. Their data also suggest that these "pores" are not modifications of specific protein channels present in the membrane but may be due to discontinuities in the membranes caused by phase separations which are induced by PC12 rich domains. Such phase separations may also induce membrane fusion (Papahadjopoulos et al., 1977; Hoekstra, 1982).

Further studies with fluorescently labeled or radiolabeled lipids, coupled with electron microscopy, are needed to determine the location of lipids added to bull sperm and to help elucidate their mehanism of action. Presently, it is clear that lipids with specific lengths of fatty acyl chains specifically induce the AR and render bull sperm capable of "fertilizing" hamster eggs in vitro. This procedure may be useful in various ways, such as fertilizing bovine eggs in vitro and in developing an in vitro penetration assay to predict the fertility of bulls (Graham and Foote, 1984).

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