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Phosphatidylcholine Enhances the Acrosomal Responsiveness of Human Sperm

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ABSTRACT: Supplementing bovine serum albumin-containing medium with phosphatidylcholine (PC) accelerated the *in vitro* development of human sperm acrosomal responsiveness. Responsiveness was assessed by exposing the sperm to progesterone. The maximum effect was produced by incubation with 100 μg PC/ml, which resulted in 40% (23–56%) (mean, 95% confidence limits) of the sperm becoming responsive to progesterone at 24 hours, compared to 23% (10–40%) of control sperm. Enhancement was apparent after as little as 6 hours of incubation *in vitro*, and the number of responsive sperm was still increasing at the last time point tested (30 hours). PC had no apparent ill effects; it did not alter the percentage of motile sperm or the percentage of sperm stained with the supravital stain, Hoechst 33258. Enhanced responsiveness required prolonged incubation in PC, because PC was not effective when it was only applied at the same time as progesterone. Lysophosphatidylcholine did not enhance acrosomal responsiveness when used at concentrations from 10 ng/ml to 100 μg/ml, indicating that the effect of PC was not due to trace amounts of lysophosphatidylcholine. PC also increased the response of sperm to the Ca^{2+}/H^{+}-exchanging ionophore, ionomycin, suggesting that PC modifies an event that is coincident with or subsequent to the rise in intracellular free Ca^{2+} that is triggered by progesterone.

Key words: Acrosome reaction, capacitation, progesterone.

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When human sperm are maintained *in vitro*, a few sperm undergo spontaneous acrosome reactions. More sperm can be induced to acrosome react when they are treated with appropriate inducers such as the zona pellucida (Cross et al, 1988), preovulatory follicular fluid (Tesarik, 1985; Suarez et al, 1986), progesterone (Osman et al, 1989), or Ca^{2+}/H^{+}-exchanging ionophores (Russell et al, 1979), among others. In most cases, however, only a minority of the sperm respond. The proportion of responsive sperm increases as sperm are incubated longer *in vitro*, but it is rarely reported to approach 50%.

Why the majority of sperm do not respond to inducers of the acrosome reaction is not clear. Perhaps sperm in a single ejaculate are physiologically different, or perhaps the incubation conditions that we use are not optimal. In any case, the limited development of acrosomal responsiveness does restrict some uses of human sperm. Here we report that including 100 μg/ml phosphatidylcholine (PC) in the incubation medium increases the number of sperm that are acrosomally responsive by about 70%, with no apparent deleterious effect.

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Materials and Methods

Materials

The following chemicals were used: bovine serum albumin (Pentex Bovine Albumin, fraction V, reagent grade, cat. no. 81-066-7, lot 46; Miles Inc., Kankakee, Illinois), fluoresceinated *Pisum sativum* agglutinin (Vector Laboratories, Burlingame, California), soybean PC, and lysophosphatidylcholine (LPC) (cat. nos. 840054 and 830072, respectively; Avanti Polar Lipids, Alabaster, Alabama). All other chemicals were from Sigma Chemical Company (St. Louis, Missouri). Ninety-six-well plates were purchased from Corning Glass Works (cat. no. 25880-96; Corning, New York).

Sperm Treatments

Ejaculates were obtained from healthy donors by masturbation and used within 1 hour. The ejaculates had normal volume, sperm motility, concentration, and morphology according to World Health Organization criteria. Motile sperm were selected by centrifuging the ejaculate on a two-layer Percoll gradient (Siiteri et al, 1988). Sperm were adjusted to 10^6/ml in incubation medium, a modified Tyrode's solution containing 26 mg/ml bovine serum albumin (Suarez et al, 1986). Aliquots of 0.075 ml were incubated in wells of a 96-well plate at 37°C in a humidified atmosphere of 5% CO_2/95% air.

The number of acrosomally responsive sperm was determined by exposing the sperm to either progesterone or ionomycin and determining acrosomal status using *Pisum sativum* agglutinin, all as previously described (Cross, 1993). (This method assesses acrosomal status of those sperm that exclude the supravital stain, Hoechst 33258.) Progesterone treatment was for 10 minutes in the presence of 0.5 μg/ml Hoechst 33258. Before treatment with ionomycin, the concentration of albumin was reduced to 3 mg/ml (Thomas and Meizel, 1988). Ionomycin was added to 4.5 μM, and at 2.5 minutes the albumin concentration was restored to 26 mg/ml. At 10 minutes, Hoechst 33258 was added to 0.5 μg/ml. At 20 minutes, the sperm were processed to determine acrosomal status. (See Cross [1993] for more details.) Triplicate samples were prepared, and 200 sperm were inspected per sample. Most experiments were performed five times (see Results).

Sperm motility was assessed by counting the percentage of motile sperm in a sample of 100 sperm and by assigning a rating of 1 to 3 for the progressiveness of sperm motion.

Lipids

PC liposomes were prepared by sonication. An aliquot containing 5 mg PC (25 mg/ml in chloroform) was dried under N_2 (at least 10 minutes, 37°C). One ml of incubation medium lacking albumin was added and warmed (10 minutes, 37°C) to hydrate the PC. The suspension was sonicated using a microprobe (Branson Model 200 Sonifier, 17 to 24 watts output) for 5 minutes with the tube immersed in water at room temperature to prevent overheating. The suspension was then centrifuged (12,000 × *g*, 5 minutes) to sediment titanium fragments that might have formed during sonication. Albumin (26 mg/ml) was added to the supernatant. Control sonicated medium was prepared beginning with an aliquot of chloroform that did not contain PC, and it was added to control sperm suspensions in place of PC. Solutions of LPC were prepared by dissolving dry LPC in incubation medium. The lipid preparations were stored at −20°C.

Statistics

Percentage data were transformed (arcsin$\sqrt{[\%/100]}$) before analysis. The paired-sample *t*-test was used to compare two means. Experiments that produced more than two means were analyzed by ANOVA using a randomized block design, with experimental date as the block. (Each experimental date employed a single ejaculate.) Tukey-Kramer pairwise comparisons were performed on the means, with $P < 0.05$ indicating significance. SYSTAT (SYSTAT, Inc., Evanston, Illinois) or InStat (GraphPad Software, San Diego, California) was used for the analyses. Results are presented below as mean (95% confidence limits).

Results

When sperm were incubated for 24 hours in control incubation medium, 6% (4–8%) of the sperm spontaneously acrosome reacted and 25% (17–35%) were observed to be reacted following a 10-minute exposure to progesterone (Fig. 1). When PC (0.01–1.0 mg/ml) was included in the incubation medium from the start of the experiment, there

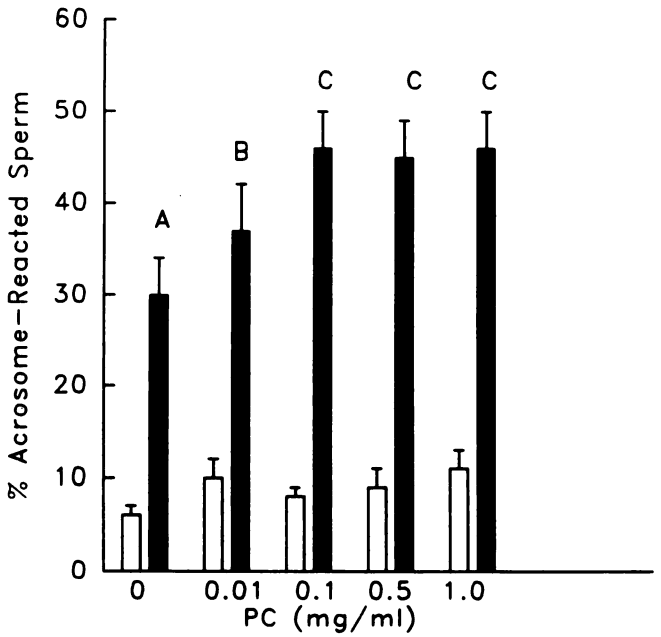

FIG. 1. Effect of phosphatidylcholine (PC) on acrosomal responsiveness. Sperm were incubated 24 hours with the indicated amount of PC in the incubation medium, and then spontaneous acrosome reactions (open bars) and the total number of reactions observed among sperm treated with progesterone (solid bars) were assessed. None of the PC treatments significantly affected the rate of spontaneous reactions ($P > 0.05$). PC increased the incidence of sperm that respond to progesterone; bars with different letter designations are significantly different from each other ($P < 0.05$). Vertical bars depict the standard error of the mean (SEM); $n = 5$.

was a very small but not significant increase in the number of spontaneously reacted sperm. In contrast, there was a large, dose-dependent increase in the number of sperm that reacted upon exposure to progesterone. The maximum response was reached at a concentration of 100 μg of PC/ml.

The difference between the percentage of sperm that spontaneously react and the total percentage of reacted sperm following exposure to progesterone is the percentage of sperm that responded to progesterone. The percentage was 23% (10–40%) among control sperm and 40% (23–56%) among sperm incubated with 100 μg/ml PC. All of the results presented below refer to this measure of progesterone-induced acrosome reactions.

To determine the earliest time at which acrosomal responsiveness was enhanced, aliquots of sperm that had been incubated in either control medium or medium containing 100 μg of PC/ml were exposed to progesterone. At 6 hours of incubation and thereafter, PC-treated sperm were significantly more responsive (Fig. 2). It is apparent from Figure 2 that the maximum effect was not reached by the end of the experiment, at 30 hours. Incubation with PC resulted in the majority of the sperm becoming responsive; at 30 hours, 58% (53–64%) of the sperm were

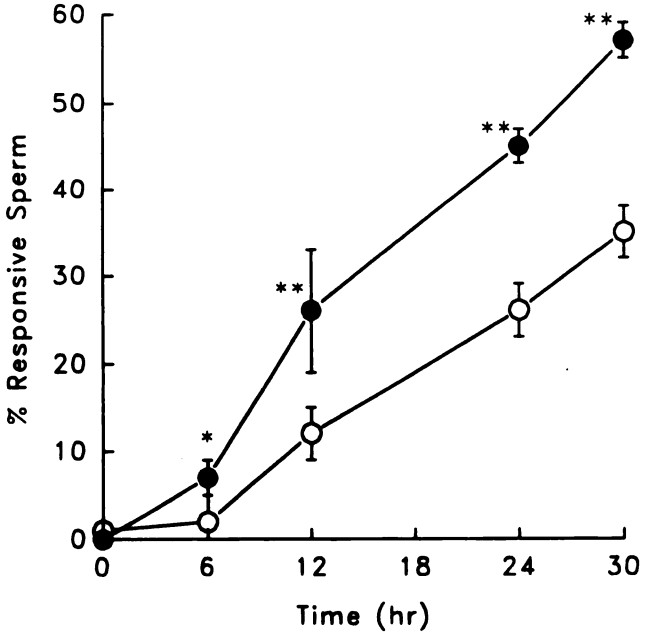

FIG. 2. Development of acrosomal responsiveness during incubation *in vitro*. Sperm were incubated in either control medium (open circles) or medium containing 100 μg PC/ml (closed circles). At intervals, the incidence of sperm that were responsive to progesterone was determined. Incubation with PC caused a significant increase in the number of responsive sperm as early as 6 hours. A single asterisk indicates $P < 0.05$ for the two treatment groups; a double asterisk indicates $P < 0.01$. The vertical bars depict SEM; $n = 5$.

responsive to progesterone. The spontaneous rate remained low: 6% (3–10%).

Incubation in PC had no apparent deleterious effect on the sperm. PC (100 μg/ml, 24 hours) did not alter the percentage of motile sperm, their progressiveness rating, or the percentage of sperm that were stained with the supravital stain, Hoechst 33258 (data not shown).

In the experiments depicted in Figures 1 and 2, the sperm were still in PC-containing incubation medium when progesterone was added. To separate the effect of prolonged incubation in PC from the possible effect of PC being present when progesterone was added, we tested whether adding PC at the time of progesterone treatment was sufficient to enhance acrosomal responsiveness. Sperm were incubated 1) with 100 μg/ml PC in the incubation medium, or 2) in incubation medium without PC. After 24 hours, the former group was treated with progesterone alone. Half of the latter group was treated with progesterone alone and half was treated with a mixture of progesterone and PC (in an amount that gave the same final concentration of PC as in the former group). Adding PC at the same time as progesterone did not enhance the response of sperm (Table 1), suggesting that sperm must be exposed to PC for a prolonged period for acrosomal responsiveness to be affected.

Table 1. *Time of PC addition*

Group	Time of PC addition	% Responsive sperm (95% confidence limits)
1	No PC	18[a] (2–45)
2	0 hours	30[b] (6–64)
3	24 hours	16[a] (4–36)

Sperm were incubated for 24 hours either in the absence of PC (Groups 1 and 3), or in the presence of 100 μg/ml PC (Group 2). At 24 hours, aliquots of the sperm suspensions were assessed for the percentage of sperm that were responsive to progesterone. PC was added as a mixture with progesterone to Group 3. Values with different superscripts are significantly different; $n = 6$.

Table 2. *Effect of LPC on acrosomal responsiveness*

LPC (μg/ml)	% Responsive sperm (95% confidence limits)
0.0	29 (9–54)
0.01	20 (1–54)
0.10	25 (15–37)
1.0	26 (12–42)
10.0	25 (9–45)
100.0	25 (15–37)

Sperm were incubated in medium containing various concentrations of LPC. At 24 hours, the number of sperm that were responsive to progesterone was determined. There were no significant differences among the six groups ($P > 0.05$, $n = 3$).

To determine if the effect was unique to progesterone, we tested the effect of incubating sperm 24 hours in incubation medium containing 100 μg PC/ml on the response of sperm to the Ca^{2+}/H^{+}-exchanging ionophore, ionomycin. PC enhanced the response to ionomycin to 33% (11–61%), compared to 13% (1–36%) among sperm in control medium ($P < 0.05$, $n = 8$).

To determine if trace amounts of LPC in the liposome suspension might account for the action of PC, we tested the effect on acrosomal responsiveness when sperm were incubated for 24 hours in incubation medium containing LPC (0.01–100 μg/ml). LPC did not increase acrosomal responsiveness at any of these doses (Table 2).

Discussion

Supplementing incubation medium with 100 μg/ml PC caused a substantial increase in the number of sperm that acrosome-react upon exposure to progesterone or ionomycin. The number of responsive sperm grew with time and did not reach a plateau by 30 hours. To the extent that acrosomal responsiveness reflects sperm capacitation, one can conclude that PC increases the number of capacitated sperm.

How PC exerts its effect is not clear from these experiments. PC did not significantly increase the rate of spon-

taneous acrosome reactions, so it is acting differently from liposomes of dilauroyl-PC, which induce acrosome reactions in bovine and human sperm (Graham et al, 1986; Holden and Trounson, 1992). The effect of PC apparently cannot be explained by the presence of LPC in the suspension. LPC is known to induce acrosome reactions of human sperm (Byrd and Wolf, 1986) and to facilitate acrosomal responsiveness of guinea pig sperm (Fleming and Yanagimachi, 1981). In our hands, however, incubation in LPC-containing medium neither induced acrosome reactions nor increased the sperm response to progesterone.

PC may be altering the lipid content of sperm membranes. Cholesterol may play a role in the regulation of the acrosome reaction and/or fertilizing ability (reviewed by Davis, 1978; Go and Wolf, 1983). PC liposomes might increase the net efflux of cholesterol from sperm by serving as a cholesterol sink in the medium. Incubating bovine sperm with liposomes made of an equimolar mixture of PC and phosphatidylethanolamine increases the number of sperm that acrosome-react upon subsequent exposure to LPC; this effect is accompanied by an efflux of sperm cholesterol (Ehrenwald et al, 1988a). Bovine sperm treated in that fashion are capable of penetrating zona-free hamster oocytes and fertilizing bovine oocytes (Ehrenwald et al, 1988b). Alternatively, PC might enter the sperm membrane. Lipid transfer protein I, which contaminates some albumin preparations (Ravnik et al, 1993), might promote the movement of PC into the sperm membrane in albumin-containing incubation medium. It seems unlikely that PC liposomes are fusing directly with the sperm plasma membrane. Arts et al (1993) found that PC-containing large unilamellar vesicles did not fuse with sperm, and we have failed to observe bulk transfer of fluorescent lipid probes from PC liposomes to sperm (data not shown). More experiments are required to understand how PC exerts its effect.

PC also enhanced the response of sperm to ionomycin, demonstrating that the effect of PC is not unique to progesterone. This observation also implies that a target for PC lies in a part of the signal transduction pathway that is shared by ionomycin and progesterone, that is, at or downstream from the rise in intracellular calcium that follows interaction of progesterone with the sperm surface (Thomas and Meizel, 1989; Blackmore et al, 1990; Blackmore and Lattanzio, 1991; Meizel and Turner, 1991). PC might, for example, increase the ability of the plasma membrane to fuse with the acrosomal membrane. Our results with ionomycin should be interpreted with caution, however. We used ionomycin under conditions that trigger acrosome reactions in 24-hour incubated sperm, but not in 0-hour sperm (Thomas and Meizel, 1988; unpublished observations). It is not yet clear that this protocol produces a calcium rise of similar magnitude under all experimental conditions. We cannot presently rule out the hypothesis that ionomycin triggers a larger calcium rise in sperm incubated with PC than in those incubated without PC, but this possibility will be simple to test. Another question that remains to be answered is whether PC enhances the response of sperm to the zona pellucida.

Our findings may have some practical applications. Studies on the mechanism of the acrosome reaction of human sperm are sometimes hindered by the small number of sperm that respond to inducers. Incubation for 24 hours with PC increases the number of responsive sperm by about 70%. By incubating sperm longer than 30 hours, it may be possible to produce sperm suspensions in which almost all of the sperm are responsive. (It remains to be seen, however, whether sperm motility can be maintained under those conditions.)

There are now many reports that acrosomal responsiveness correlates with the fertility of an ejaculate (e.g., Calvo et al, 1989; Cummins et al, 1991; Fenichel et al, 1991; Marshburn et al, 1991; Pampiglione et al, 1993). If diminished acrosomal responsiveness contributes to some cases of subfertility, then increasing the responsiveness of sperm of those men may improve their fertilizing ability. PC may be useful in this regard.

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