Receptor activity of the hamster and mouse solubilized zona pellucida before and after the zona reaction

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Summary. Capacitated golden hamster spermatozoa bound to the inner as well as to the outer surface of the zona pellucida, suggesting that the receptor-for-spermatozoa may occur throughout this egg envelope. When solutions of hamster zonae pellucidae were prepared by heating zonae in an aqueous buffer, receptor activity was retained and was stable to boiling. The addition of such solutions to capacitated spermatozoa (5 zonae/ μ l) prevented them from binding to eggs and fertilizing them. Solubilized mouse zonae were also partly effective, as would be predicted from the limited cross-binding between mouse and hamster gametes. Receptor activity was lost following the zona reaction.

Introduction

Little is known concerning the nature and distribution of the receptor in the zona pellucida of the mammalian egg to which the spermatozoa must bind to achieve fertilization. Using golden hamster gametes *in vitro* we have previously established that the zona pellucida retains its sperm-binding property after exposure for 30 min to 60°C, a temperature close to its melting point (Gwatkin, Williams & Andersen, 1973a). This binding property is also stable to a number of glycosidases and lipases, but is inactivated by low concentrations of trypsin or chymotrypsin (Hartmann & Gwatkin, 1971; Gwatkin *et al.*, 1973a) and by a trypsin-like protease which produces the zona reaction (Gwatkin, Williams, Hartmann & Kniazuk, 1973b). We have now extended these studies.

Materials and Methods

Eggs were collected, approximately 20 h after the HCG injection, from the ampullae of 6–8-week-old mice and golden hamsters induced to superovulate. The cumuli oophori were removed by incubation in hyaluronidase as described by Gwatkin, Andersen & Hutchison (1972) and the zonae pellucidae were isolated by forcing the eggs through a micropipette with an inner tip-diameter of 50 μ m.

To determine the extent of sperm binding to the inner surface of the zona pellucida, the zonae were fragmented by vigorous pipetting (inner diameter of pipette tip, 25 μ m) and pieces roughly equivalent to one-half of an intact zona were selected for study. Groups of 20 such $\frac{1}{2}$ -zonae were incubated for 60 min in phosphate-buffered saline (PBS, pH 7.4; Dulbecco & Vogt, 1954) and in PBS containing crystalline trypsin (1.0 μ g/ml, 3 times crystallized: Worthington Biochemical Corp., Freehold, New Jersey). The zona fragments were then washed and placed in 20 μ l drops of Medium 199M2 (Gwatkin & Andersen, 1973). Then an equal volume of capacitated epididymal sperm suspension (approximately 10^7 motile cells/ml) was added. Capacitation of the spermatozoa was achieved by incubation for 5 h with cumulus cells, as described previously (Gwatkin *et al.*, 1972).

After incubation of the zona fragments with the spermatozoa on a rocker (5–6 oscillations/min) for 90 min at 37.5°C in an atmosphere of 5% CO₂ in air, the fragments were again washed by transferring them through 3 successive 0.5 volumes of Medium 199M2 and mounted in a 1–2 μ l drop of medium on a microscope slide. Using a phase-contrast microscope, the number of motile sperm-

atozoa bound to the inner (concave) and outer (convex) surfaces was recorded. Juxtaposed, but possibly unbound, spermatozoa were excluded from the count by restricting the scoring to motile spermatozoa.

Zona solutions were prepared by suspending 1000 or 2000 isolated zonae pellucidae in 50 or 100 μ l PBS, respectively, contained in 0.5 ml glass centrifuge tubes. The tubes, covered with paraffin film and again with aluminium foil, were incubated for 35 min in a water-bath maintained at 65°C. The resulting zona solutions were cooled, serially diluted in PBS and 10 μ l of each dilution were added to 30 μ l capacitated sperm suspension. After incubation for 30 min, 10 cumulus-free eggs were added to each drop and their penetration by spermatozoa was recorded 90 min later, as described previously (Gwatkin *et al.*, 1972).

Zona solutions were also prepared from eggs which had undergone a zona reaction. This reaction was induced by electrical stimulation (150 V for 1 msec: Gwatkin *et al.*, 1973b) or by fertilization *in vivo* and cleavage to 2-cell embryos. The latter were obtained by treating 6–8-week-old female hamsters i.p. with 5 i.u. PMSG, followed 72 h later by 5 i.u. HCG and the introduction of fertile males. On the following day, the animals were observed for the presence of spermatozoa in the vagina and 2 days later 2-cell embryos were flushed from the oviducts.

Results

Table 1 shows that numerous hamster spermatozoa bound to the inner surface as well as to the outer surface of the zona pellucida. Pretreatment of the $\frac{1}{2}$ -zonae with trypsin (1 μ g/ml) markedly reduced binding to the outer surface and completely prevented binding to the inner surface.

Table 1. The effect of pretreatment with trypsin on the binding of hamster spermatozoa to the inside and outside of the hamster zona pellucida

Exp.	Pretreatment with trypsin	Mean no. of motile sperm. bound by $\frac{1}{2}$ -zonae*	
		Inside	Outside
1	None	26	26
	1 μ g/ml for 60 min	0	4
2	None	12	> 30
	1 μ g/ml for 60 min	0	2

* Twenty $\frac{1}{2}$ -zonae per treatment.

The binding of hamster spermatozoa to the outer surface of mouse zonae pellucidae was extremely low (approximately 1 spermatozoon per 10 $\frac{1}{2}$ -zonae), as shown in Table 2. However, binding to the inner surface, although less than to the corresponding surface of the hamster zona, was appreciable (approximately 2 spermatozoa per $\frac{1}{2}$ -zona).

Table 2. The binding of capacitated hamster spermatozoa to mouse zonae pellucidae

Origin of zonae	No. of $\frac{1}{2}$ -zonae per exp.	Mean no. of motile sperm. bound per $\frac{1}{2}$ -zona	
		Inside	Outside
Mouse	15	2.47	0.07
	33	1.97	0.15
Hamster	15	5.07	9.73
	36	10.33	> 15

When 2000 zonae were incubated in 100 μ l PBS for 35 min at 65°C, the zonae dissolved and on cooling to room temperature remained in solution. The addition of such zona preparations to suspensions of capacitated hamster spermatozoa had no detectable effect on their motility but blocked their ability to bind to eggs or to fertilize them. A final concentration of 1 solubilized zona/ μ l reduced the percentage of eggs penetrated by approximately 50% (Text-fig. 1). Penetration was prevented completely by 5 solubilized zonae per μ l. Exposure of the eggs alone to the zona solution had no effect on subsequent penetration by spermatozoa, showing clearly that its action was on the male gametes. Storage of hamster zona solutions for 1 week at 5°C, freezing and thawing or boiling for 5 min had no effect on their activity.

Text-fig. 1. Effect on the proportion of eggs penetrated of varying the concentration of solubilized hamster zonae pellucidae added to a capacitated sperm suspension. The closed and open circles represent two independent experiments.

Table 3 shows that mouse zona solutions, prepared by heating 1000 zonae to 65°C for 35 min in 50 μ l PBS, produced only a partial block to sperm penetration. When zona solutions were prepared from hamster eggs 30 min after electrical stimulation with 150 V for 1 msec, when only a fraction of the eggs should have undergone a zona reaction (Gwatkin *et al.*, 1973b), a similar partial block was produced. However, solutions prepared 2 h after electrical stimulation or from 2-cell embryos were completely inactive.

Table 3. Effect of solubilized mouse zonae and hamster zonae which have undergone a partial or complete zona reaction on the fertilization of hamster eggs *in vitro*

Source of solubilized zonae*	% Eggs penetrated†	
	Exp. 1	Exp. 2
None	100	100
Hamster eggs	0	0
Mouse eggs	30	40
Hamster eggs, 30 min after electrical stimulation	20	30
Hamster eggs, 2 h after electrical stimulation	100	100
2-Cell hamster embryos	100	100

* See text.

† Ten eggs per treatment.

Discussion

These studies demonstrate that the capacitated hamster spermatozoon forms a tight bond, not only with the outside of the zona pellucida, but with its inner surface as well. It seems probable that the trypsin-sensitive receptor-for-spermatozoa (Hartmann & Gwatkin, 1971) is present throughout the zona, where it would be expected to maintain gamete association as the spermatozoon burrows through to reach the vitellus. However, there may be a gradient, with the greatest concentration of receptor towards the outer surface, and this could account for the apparently greater sensitivity of the inner-surface binding to trypsin (Table 1).

The slight binding of hamster spermatozoa to the outer surface of the mouse zona is consistent with earlier observations that such binding is essentially species-specific (Hartmann, Gwatkin & Hutchison, 1972). Why a greater number of hamster spermatozoa bind to the inner than to the outer surface of mouse zonae is not known. Possibly the receptor on the outer surface is partly masked by non-zona components derived from the cumulus or oviduct. Fox & Shivers (1975) have demonstrated that hamster eggs bind oviductal antigens on the outside of their zonae. Experiments are in progress to determine whether greater numbers of spermatozoa bind to the external surface of the zona pellucida when eggs are obtained directly from the follicle rather than from the oviduct.

Our experiments also demonstrate that the receptor activity of the zona pellucida can be prepared in a soluble form by heating. Mouse zonae have been observed to melt at 68–70°C, but receptor activity of the solution was not determined (Cholewa-Stewart & Massaro, 1972). Experiments are in progress to determine whether low pH, which is known to dissolve the zona pellucida of the rat and mouse eggs (Hall, 1935; Gwatkin, 1964) and presumably produces a conformational change in the zona protein, will also yield active solutions. Similar experiments are also in progress with 2-mercapto-ethanol, which dissolves the zonae pellucidae of mouse and rabbit eggs by disrupting S–S cross-links in the zona protein (Inoue & Wolf, 1974; Gould, Zaneveld, Srivastava & Williams, 1971).

This is the first time that a soluble form of the receptor-for-spermatozoa has been prepared from mammalian eggs. However, a soluble sperm-binding protein has been partly purified from the vitelline membrane of sea-urchin eggs (Aketa, Tsuzuki & Onitake, 1968; Aketa, 1973). The receptor is inactivated by trypsin (Aketa, Onitake & Tsuzuki, 1972) and by disulphide-reduction (Aketa & Tsuzuki, 1968). The partial effectiveness of solubilized mouse zonae in blocking the fertilization of hamster eggs *in vitro* is consistent with the limited degree of binding which occurs between hamster spermatozoa and mouse zonae and suggests that the receptors on the zonae of these species share a common determinant.

As a result of the zona reaction, induced either by fertilization or by electrical stimulation, the capacity of the solubilized zonae to prevent fertilization *in vitro* is lost. This change probably results from the cleavage of arginyl- or lysyl-bonds, since a trypsin-like protease discharged from the cortical granules has been implicated in the zona reaction (Gwatkin *et al.*, 1973b).

Since the zona pellucida contains carbohydrate (Braden, 1952; Stegner & Wartenberg, 1961; Oakberg & Tyrrell, 1975) the receptor could be a glycoprotein. Several sugar-binding plant agglutinins (lectins) have been observed to bind to the zonae of hamster eggs (Oikawa, Nicolson & Yanagimachi, 1974). One of these, wheat germ agglutinin (WGA), blocks fertilization *in vitro* and it has been suggested that the WGA receptor may be near, or even structurally related to, the receptor-for-spermatozoa (Oikawa, Yanagimachi & Nicolson, 1973; Nicolson, Yanagimachi & Yanagimachi, 1975). However, it should be borne in mind that some lectins can block fertilization by inducing a cortical reaction (Gwatkin, Rasmusson & Williams, 1976) and lectins may also bind to adsorbed non-zona substances. Furthermore, the resistance of the receptor-for-spermatozoa to glycosidases argues against a critical role for terminal sugars as receptor determinants.

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