BIOLOGY OF REPRODUCTION 17, 350–354 (1977)

Induction of Zonal and Egg Plasma Membrane Blocks to Sperm Penetration in Mouse Eggs with Cortical Granule Exudate

DON P. WOLF and MITSUMA HAMADA

Division of Reproductive Biology,
Department of Obstetrics and Gynecology, and
Department of Biochemistry and Biophysics,
University of Pennsylvania School of Medicine,
Philadelphia, Pennsylvania 19174

ABSTRACT

The contents of cortical granules (cortical granule exudate = CGE) were recovered from zona-free mouse eggs inseminated *in vitro* with capacitated epididymal spermatozoa. Preincubation of cumulus-free, zona-intact eggs in CGE led to reduced penetration levels upon insemination. This reduction was CGE concentration- and time-dependent and was susceptible to inhibition by soybean trypsin inhibitor and by p-aminobenzamidine. Control experiments eliminated active contributions from the sperm suspension used to elicit granule release. CGE was also active in reducing penetration of zona-free eggs. These results indicate that cortical granule contents are capable of modulating sperm penetration of mouse eggs at both the zona pellucida and at the egg plasma membrane.

INTRODUCTION

Two mechanisms are recognized by which mammalian eggs can modulate their own receptivity to sperm, namely, the zona reaction and the egg plasma membrane block to polyspermy (Austin, 1965). Presumably, a penetrating sperm initiates these events by triggering the release of the egg's complement of cortical granules and the blocks are subsequently effected through CGE-induced modifications of either the zona or the egg plasma membrane. Evidence for the existence of a zona reaction in mammalian eggs was first presented in 1954 (Braden et al., 1954), based on disparities in the observed and predicted frequencies of penetrating sperm seen in eggs recovered from natural matings. Subsequently, Barros and Yanagimachi (1971) provided direct experimental evidence for the existence of a zonal block to sperm penetration of hamster eggs by demonstrating cortical granule exudate-dependent reductions in sperm binding to and penetration of zona-intact eggs. More recently, Gwatkin et al. (1973) have characterized hamster cortical granule exudate activity as trypsin-like, based on its susceptibility to trypsin inhibitors.

Evidence for the existence of an egg plasma membrane block to polyspermy was derived originally from the observation that monospermic embryos recovered from natural matings contained supplemental sperm in the perivitelline space (Austin and Braden, 1953; Braden et al., 1954). Direct experimental evidence is now available supporting the existence of a block in zona-free hamster (Barros and Yanagimachi, 1972) and mouse eggs (Wolf, 1977); however, a role for cortical granule exudate in this process remains speculative.

We have undertaken preliminary characterization of cortical granule exudate released from zona-free mouse eggs. Our findings indicate that mouse exudate, similar to that of the hamster, is capable of inducing both a zona reaction and a plasma membrane block to sperm penetration in zona-intact and zona-free mouse eggs, respectively. A preliminary account of this work has been presented (Wolf and Hamada, 1977).

MATERIALS AND METHODS

Tubal eggs were collected from superovulated Swiss mice (Taconic Farms, Tarrytown, N.Y.) and freed of their cumulus in a modified Krebs-Ringer bicarbonate medium containing 2 percent bovine serum albumin (Sigma, Fraction V), as described previously (Inoue and Wolf, 1974; Wolf and Inoue, 1976). Epididymal sperm were capacitated by preincubation in this medium for 60–120 min. Zonae were removed from cumulus-free eggs manually (Wolf et al., 1976). Cortical granule release was effected by inseminating 200 zona-free eggs in 50 μl drops for 45 min at

Accepted April 27, 1977.
Received February 25, 1977.

37° with 2 × 10^5 capacitated epididymal sperm/ml, unless indicated otherwise. Previous studies served to document the release of granules from these penetrated zona-free eggs (Wolf et al., 1976; Wolf and Nicosia, unpublished results). Gametes were collected by centrifugation (480 × g, 10 min at 4°), and the supernatant solution (cortical granule exudate: CGE) was removed. CGE was divided into two 20 μl drops under Dow-Corning 200 Fluid (50 cs) in insemination dishes; test eggs were added, followed 30 or more minutes later by capacitated sperm (2 × 10^5 capacitated cells/ml). Alternatively, eggs were preincubated with CGE under identical conditions, then washed thoroughly and placed in 200 μl drops of fresh culture medium before insemination. After 2–4 h additional incubation, penetration and fertilization were scored on aceto-lacmoid stained whole mounts, as described previously (Wolf and Inoue, 1976; Wolf et al., 1976). Penetration included all sperm that had passed through the zona (supplemental sperm, as well as sperm within the vitellus), while fertilization denoted only sperm within the vitellus. The statistical significance of the results was evaluated by the Chi test for equality of two independent proportions (Ipsen and Feigl, 1970).

RESULTS

Cortical granule exudate (CGE) released from zona-free eggs was capable of decreasing capacitated sperm penetration of cumulus-free, zone-intact eggs (Table 1). While both penetration and fertilization levels are included here for comparative purposes, the best quantitative indicator of sperm-egg interaction is the mean number of penetrating sperm per inseminated ovum. In the experiments reported in Table 1, control ova were placed in 20 μl drops and inseminated immediately, while CGE-treated eggs were preincubated under identical conditions for one hour prior to insemination. In the third treatment group (sperm supernatant solution), eggs were preincubated for one hour in 20 μl drops of medium containing only sperm-derived components, i.e., sperm supernatant solutions were prepared under conditions that mimicked those employed in CGE preparation and zona-free eggs were omitted. Aging could be eliminated as a contributing factor to CGE-dependent reductions in penetration since eggs treated with sperm supernatant solutions were also preincubated. While these results implicate the eggs as a source of CGE activity, one further experiment was conducted to evaluate possible sperm-related contributions. Such an experiment was considered essential, since proteolytic activities are undoubtedly involved in these phenomena (Gwatkin et al., 1973); sperm contain the trypsin-like enzyme, acrosin; and release of acrosin *in vitro* may require the presence of eggs, i.e., induction of the acrosome reaction may be egg-dependent. Accordingly, a high and a low sperm concentration were employed to effect cortical granule release from zona-free ova, and the resultant CGE activity was monitored against zona-intact ova (Table 2). A 45 and 48 percent reduction in penetration (based on mean numbers of penetrating sperm) was observed when CGE was prepared with 5 × 10^7 and 5 × 10^4 sperm/ml, respectively, demonstrating that CGE activity is independent of the sperm suspension over this concentration range.

CGE-induced reductions in zonal penetration were related to the concentration of CGE (Fig. 1). Results from two experiments were similar and have therefore been combined (313 eggs); 260 or 200 zona-free eggs were utilized as the source of CGE. CGE was labile at 37°, as judged by kinetic experiments where maximum reductions in sperm penetration of zona-intact

TABLE 1. Cortical granule exudate-dependent reduction in sperm penetration of zona-intact mouse eggs.

Treatment	Penetration, %	Fertilization, %	Mean number penetrated sperm/ inseminated ovum
Control	63 (51/81)	51 (41/81)	1.00
CGE	*24 (20/82)	*15 (12/82)	0.28
Sperm supernatant solution	62 (34/55)	56 (31/55)	0.78

*Significantly reduced over controls (P<0.01).

Summation of 5 separate experiments in which CGE was recovered from 96-228 zona-free eggs. Eggs were preincubated for 1 h in test solutions and inseminated directly.

TABLE 2. Sperm concentration and cortical granule exudate activity against zona-intact ova.

Sperm concentration (cells/ml)		Penetration, %	Fertilization, %	Mean number penetrated sperm/ inseminated ovum
5×10^7	Control	76 (178/234)	72 (168/234)	1.54
	CGE	*52 (171/332)	*42 (141/332)	0.84
5×10^4	Control	78 (134/173)	73 (126/173)	1.14
	CGE	*42 (67/160)	*38 (61/160)	0.56

*Significantly different from control ($P<0.01$), but not from each other ($0.1<P>0.05$).

Summation of 7 separate experiments. Eggs were preincubated for 30 min in test solutions and inseminated directly.

eggs were seen within the first hour of preincubation. The mean number of penetrated sperm/inseminated egg dropped from 0.71 in the control to 0.26, 0.26 and 0.22 after 1, 2 and 3 h of preincubation in CGE, respectively. In these experiments, eggs were preincubated with CGE, washed and subsequently inseminated in fresh culture medium. Thus, this experiment also eliminates the possibility that CGE might act directly on capacitated sperm in the insemination reaction, since the two were never in contact. Based on the probability that CGE activity involves a trypsin-like enzyme, the influence of trypsin inhibitors was examined (Table 3). Both soybean trypsin inhibitor (2.5 mg/ml) and the synthetic trypsin inhibitor, p-aminobenzamidine (500 μg/ml), when present during preincubation, blocked the CGE-dependent reduction in sperm penetration of zona-intact eggs.

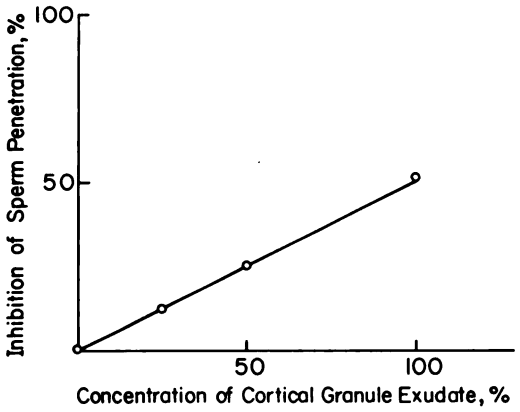

FIG. 1. Cortical granule exudate concentration dependency in the reduced sperm penetration of zona-intact eggs. CGE was diluted with culture medium to produce 25 and 50 percent solutions, and the degree of inhibition was based on differences in the mean number of penetrating sperm per inseminated egg.

As indicated in the Introduction, a cortical granule involvement in the establishment of an egg plasma membrane block to sperm penetration has been postulated, although direct experimental evidence is lacking. To examine this possibility in the present study, the activity of CGE was monitored against zona-free eggs (Table 4). Statistically significant reductions were seen in sperm incorporation into CGE-pretreated, zona-free eggs, indicating that CGE is capable of modulating sperm penetration at the egg plasma membrane.

DISCUSSION

We conclude from the present study that mouse egg cortical granules contain a trypsin-like activity capable of modulating sperm penetration of the zona pellucida and the egg plasma membrane. Our evidence is similar to that available in the hamster (Gwatkin et al., 1973; Gwatkin and Williams, 1974), and includes the demonstration of: a) a direct relationship between CGE concentration and the extent of penetration reduction, and b) the susceptibility of CGE activity to trypsin inhibitors. However, it is important to note that in these experiments CGE-dependent reductions were not always statistically significant. Such results may reflect incomplete release of CGE from inseminated zona-free eggs or loss of activity during

TABLE 3. The effect of trypsin inhibitors on cortical granule exudate-dependent zonal block.

Preincubation conditions	Penetration, %	Fertilization, %	Mean number penetrated sperm/ inseminated ovum
Culture medium control	77 (17/22)	64 (14/22)	1.27
CGE	*57 (30/53)	*43 (23/53)	0.57
CGE + SBTI (2.5 mg/ml)	77 (34/44)	66 (29/44)	0.87
Culture medium control	77 (231/301)	72 (217/301)	1.17
CGE	*47 (174/369)	*40 (146/369)	0.65
CGE + pAB (500 μg/ml)	68 (199/295)	63 (185/295)	0.97

147 and 200 zona-free eggs were used in the SBTI and pAB experiments, respectively.

Eggs were preincubated for 30 min in test solutions, washed 3X in fresh culture medium and inseminated.

*Significantly reduced over controls ($P \leq 0.05$).

subsequent experimental manipulation. Indeed, kinetic experiments indicated that CGE activity was labile at 37°, and preliminary studies suggest that activity may also be lost upon centrifugation. In any case, we never observed complete inhibition of penetration, as reported by Gwatkin et al. (1973) with CGE pretreated hamster eggs. This disparity may simply reflect CGE concentration differences: 600 eggs/40 μl vs. 200 eggs/50 μl employed here. We chose to retain the lower concentration for practical reasons, with the objective of defining stable *in vitro* conditions for CGE activity. These CGE concentrations are approximately 10^3-fold lower than those anticipated *in vivo*, assuming that in the latter case CGE will disperse throughout the volume of the perivitelline space and zona pellucida. Thus, *in vitro*, the CGE contents of a single egg are discharged into 2.5×10^{-4} cc, while the combined volumes of zona and perivitelline space (volume of sphere with 90 μ diameter—volume of sphere with 65 μ diameter) are approximately 10^{-7} cc.

TABLE 4. Cortical granule exudate-induced reduction in sperm penetration of zona-free mouse ova.

	Fertilization, %	Mean number penetrated sperm/ inseminated ovum
Control	100 (53/53)	1.34
CGE	*79 (68/86)	0.98

*Significantly reduced over control ($P<0.01$).

In our study, cortical granule release was triggered with sperm, and experiments were conducted which excluded possible sperm contributions to CGE activity. Such controls were unnecessary in the hamster work, since granule release was elicited electrically (Gwatkin et al., 1973). Recently, granule release *in vitro* in response to electrical stimulation has also been documented in the mouse (Gulyas, 1976; Zamboni et al., 1976), and it should be interesting to examine the properties of mouse CGE obtained in this manner.

The dependence on a zona versus an egg plasma membrane block to multiple sperm penetration in mammalian eggs is species-specific. The rabbit does not show a functional zona reaction and must therefore rely on the egg plasma membrane block to maintain the monospermic condition (Austin, 1965). In contrast, hamster eggs *in vivo* show a highly efficient zona reaction and may not complete a plasma membrane block until several hours after penetration (Barros and Yanagimachi, 1972; Usui and Yanagimachi, 1976). Mouse eggs are capable of a moderately effective zona reaction (Braden et al., 1954), in addition to a rapid and effective plasma membrane block (Wolf et al., 1976; Wolf, 1977). At the molecular level, such species specificity may reflect differences in both enzyme(s) and substrate(s), i.e., in CGE activities and in zona or egg plasma membranes. Significant species-specific differ-

ences in zonae structure and composition have been surmised on the basis of the unique solubility properties of these structures, including their sensitivity to proteolytic alteration (for summary see Inoue and Wolf, 1975a). Indeed, hamster sperm but not mouse sperm binding to the homologous zona pellucida is susceptible to tryptic disruption, while the opposite situation obtains for the binding to or penetration of the homologous egg plasma membrane (Hartmann and Gwatkin, 1971; Inoue and Wolf, 1975b; Yanagimachi et al., 1976; Wolf et al., 1976). However, inconsistencies are apparent in the results even when species specificity is eliminated. For instance, assuming that CGE is a single, trypsin-like protease and given the observation that sperm binding to the mouse egg plasma membrane is more susceptible to trypsin interruption than is binding to the zona, one could predict the relative effectiveness of CGE-induced penetration blocks, i.e., that CGE would be more effective at the egg plasma membrane level. However, this prediction was not verified in the present study. Perhaps these observations should serve simply to define the status of the problem, for while multiple CGE proteases and at least one carbohydrase have clearly been defined in sea urchins (Epel et al., 1969; Carroll and Epel, 1975), direct experimental evidence defining any specific activities in mammalian CGE is still lacking.

ACKNOWLEDGMENTS

The authors thank Ms. Pat Park for secretarial assistance and Dr. Edward Carroll, Jr. for a critical review of the manuscript. This work was supported by USPHS HD-07635 and Ford Foundation 65-58B grants.

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