THE ZONA REACTION OF HAMSTER AND MOUSE EGGS: PRODUCTION *IN VITRO* BY A TRYPSIN-LIKE PROTEASE FROM CORTICAL GRANULES

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Summary. The cortical granules of hamster ova were ruptured *in vitro* by electrical stimulation (standard treatment was 150 V, 1.0 msec). Approximately 8 min were required for the cortical granule material so released to induce a complete zona reaction. The active material from the cortical granules was released into the medium from vitelli and was shown to act directly on the zona pellucida and on mouse, as well as on hamster, ova, i.e. its action is not species specific. The action of the cortical granule material was reversed by trypsin inhibitors. Such a trypsin-like protease, causing the zona reaction, was also released from vitelli by spermatozoa.

INTRODUCTION

The zona reaction, a sperm-induced change in the zona pellucida which blocks further sperm penetration into the eggs, was first described in mammals 18 years ago by Braden, Austin & David (1954). Cortical granules were reported to disappear from the surface of the vitellus and it was surmised that breakdown of these granules released a substance which rendered the zona pellucida impenetrable to spermatozoa (Austin & Braden, 1956). Recently, Barros & Yanagimachi (1971) strengthened this hypothesis by blocking the fertilization of hamster eggs with cortical granule material released from the vitellus by sperm penetration. The nature of this agent and how it might act, however, were not determined.

In this paper, evidence is presented to show that cortical granule material from hamster vitelli, released into the medium either by electrical stimulation or by spermatozoa, induces the zona reaction by means of a trypsin-like protease. This is consistent with our recent demonstration that capacitated hamster spermatozoa adhere to the zona pellucida by a receptor which is sensitive to trypsin (Hartmann & Gwatkin, 1971).

MATERIALS AND METHODS

Eggs were obtained as described previously (Gwatkin, Andersen & Hutchison,

1972) from the oviducts of superovulated golden hamsters, *Mesocricetus auratus*, 17 to 20 hr after an injection of HCG. The cumulus oophorus was removed by a 5-min exposure to hyaluronidase (450 USP units/ml Dulbecco's phosphate-buffered saline). To prepare vitelli, the zona pellucida was removed with pronase (Gwatkin, 1971). Isolated zonae pellucidae were obtained by pipetting the eggs rapidly back and forth by means of a micropipette with an orifice slightly too small to accommodate an egg (Gwatkin, 1966). Spermatozoa were obtained from the cauda epididymidis (Gwatkin & Williams, 1970) and capacitated *in vitro* with oviduct contents (Gwatkin *et al.*, 1972).

Eggs and vitelli were electrically stimulated in 10- or 20- μ l drops of medium 199M (Gwatkin & Williams, 1970) under mineral oil on the bottom of a plastic Petri dish (6 cm diameter), by means of electrodes made from No. 30 gauge needles. The stimuli were obtained either from a charged capacitor, or a stimulator (Grass Instruments, Quincy, Mass., U.S.A.) set to deliver monophasic square wave pulses of varied amplitude and duration.

Cortical granules were observed by mounting the eggs on a slide, compressing them slightly beneath a vaseline-edged cover glass and examining the uppermost surface with a phase-contrast microscope equipped with an oil-immersion objective. Resistance of the treated eggs to penetration by capacitated spermatozoa was determined by adding an equal volume of sperm suspension (1 to 3×10^7 motile spermatozoa/ml) to the 20- μ l drops containing the eggs. The dish containing the drops was incubated at 37° C on a rocker (five to six oscillations/min) for 90 min. The eggs were then transferred to a slide and examined for passage of sperm heads through the zona pellucida and sperm penetration into the vitellus as described previously (Gwatkin *et al.*, 1972).

RESULTS

Both capacitor discharges and monophasic square wave pulses were administered to groups of twenty eggs in 20- μ l drops of medium under oil, by means of electrodes placed directly into the drops on either side of the eggs. After incubation at 37° C for 60 min, capacitated spermatozoa were added in an equal volume of medium. Adherence of the spermatozoa and their penetration through the zona pellucida were scored 90 min later. The results are summarized in Table 1.

A 0.5- μ F and a 60- μ F capacitor, charged to 50 and 10 V, respectively, and discharged across the eggs were sufficient to block sperm entry. The latter also blocked completely the adherence of spermatozoa to the eggs. A 2.0- μ F capacitor charged to 150 V caused necrosis of the eggs, manifested by a brownish discoloration and expansion of the vitellus. Square wave pulses of 40 V with 1.0 msec duration and 50 V with 0.1 msec duration also blocked penetration. Adherence was not blocked completely until 150 V were delivered for 1.0 msec. Prolonged stimulation (20 V for 100 msec or 40 V for 50 msec) produced necrosis.

Capacitor discharges (20 V, 60 μ F) through the medium alone, before the addition of ova, did not affect sperm adherence or penetration, nor did the same stimulation of isolated zonae pellucidae affect the adherence of sperma-

Table 1. Effect of electrical stimulation of hamster eggs on adherence and penetration of capacitated spermatozoa

Electrical stimulation			Appearance of eggs	Percentage of eggs with:	
Voltage (V)	Capacitance (μF)	Duration (msec)		Adherent sperm.	Penetration by sperm.
0			Normal	100	100
50	0.1		Normal	100	100
50	0.5		Normal	10	0
150	0.5		Normal	20	0
150	2.0		Necrotic		
5	60.0		Normal	100	100
10	60.0		Normal	0	0
20	60.0		Normal	0	0
40	60.0		Normal	0	0
20		1.0	Normal	100	100
20		50.0	Normal	20	0
20		100.0	Necrotic		
40		1.0	Normal	50	0
40		50.0	Necrotic		
10		0.1	Normal	100	80
50		0.1	Normal	35	0
150		0.1	Normal	29	0
150		1.0	Normal	0	0

TEXT-FIG. 1. The rate of disappearance of cortical granules from the vitelline surface compared with the rate of acquired resistance by golden hamster ova to penetration by spermatozoa.

tozoa to them. Thus, the electrical stimulation appeared to be acting through the vitellus. The rate of disappearance of cortical granules from the vitelline surface was next examined by oil-immersion phase-contrast microscopy and compared with the rate of loss in sperm penetration (Text-fig. 1). Approximately 8 min were required for 50% of the eggs to lose cortical granules, while approximately 16 min were required for 50% of the eggs to resist sperm entry. Hence, it would appear that a period of about 8 min is needed for the cortical granule product to produce the zona reaction. Approximately 60 min were required for the entire group of eggs to release their cortical granules and to undergo a complete block to sperm entry.

The possibility of releasing a sufficient quantity of material from the cortical granules so that it could be assayed on other eggs was also explored. After removal of the zona pellucida with pronase, groups of 150 washed vitelli were placed in 10- μ l drops, and 50% of the drops were electrically stimulated (150 V for 1 msec) while the other 50% served as controls. To avoid possible heat inactivation of the cortical granule product, the cultures were incubated at

Table 2. Induction of the zona reaction by electrically stimulated vitelli

Test object	Treatment of vitelli	Sperm adherence		Sperm penetration	
		% ova or zonae with sperm. adherent	Mean no. of sperm. adherent /ovum or zona	% ova penetrated	Mean no. of sperm./ovum
Hamster ova	Unstimulated	100	3.2	100	2.7
	Stimulated	0	0	0	0
Hamster zonae pellucidae	Unstimulated	100	21.0		
	Stimulated	0			
Mouse ova	Unstimulated	75	2.6	30	1.0
	Stimulated	0	0	0	0

room temperature (22° C). After 60 min, during which the cortical granules of the stimulated eggs were observed to rupture, hamster eggs, isolated hamster egg zonae pellucidae and mouse eggs were added to the treated and to the control drops. The drops were then incubated again at room temperature for an additional 120 min. At the end of this period, the eggs and zonae pellucidae were washed (by transferring them through three 0.5-ml vols of medium) and exposed to capacitated spermatozoa. Table 2 shows that cortical granule material acted directly on the zona pellucida, altering it so that capacitated spermatozoa could not adhere. The effect is clearly not species specific, since hamster cortical granule material was effective on mouse eggs.

For the reasons mentioned in the introduction to this paper, it appeared likely that the active factor from the cortical granules was a trypsin-like protease. The effect of trypsin inhibitors on the active material released by electrically stimulated vitelli (150 V for 1 msec) was therefore determined. The inhibitors were added to the drops just before adding the test eggs. Both soybean trypsin inhibitor and *p*-aminobenzamidine, a low molecular weight trypsin inhibitor (Mares-Guia & Shaw, 1965), effectively blocked the action of cortical

granule material (Table 3). These data indicate that the active factor from cortical granules is a trypsin-like enzyme.

To determine whether the factor obtained by electrical stimulation was the same as that released on sperm entry, groups of 600 vitelli were placed in 20- μ l drops and combined with equal volumes of either capacitated spermatozoa (2×10^7 motile spermatozoa/ml) or medium. Parallel drops were prepared containing sperm suspension and medium alone. After 60 min, the contents of these drops were transferred to a test-tube and centrifuged at 1000 g for 10 min. The supernatants (confirmed free of vitelli and spermatozoa by

Table 3. Effect of trypsin inhibitors on the zona reaction in hamster eggs induced by electrically stimulated vitelli

Enzyme inhibitor		Percentage of ova	
Type	Conc. (μ g/ml)	With sperm adherent	Penetrated by sperm.
None		5	5
Soybean	100	80	65
	500	100	75
<i>p</i> -Aminobenzamidine	20	75	65
	100	80	65

Table 4. Effect of soybean trypsin inhibitor on the zona reaction in hamster eggs induced by cortical granule product released from vitelli by spermatozoa

Material tested for induction of zona reaction	Sperm adherence		Sperm penetration	
	% ova with sperm. adherent	Mean no. of sperm. adherent/ovum	% ova penetrated	Mean no. of sperm./ovum
Supernatant from sperm.	100	5.3	100	2.5
Supernatant from vitelli, unexposed to sperm.	100	3.4	100	2.6
Cortical granule product	0	0	0	0
Cortical granule product + Soybean trypsin inhibitor (1 μ g/10- μ l drop)	100	5.2	100	2.2

microscopic examination) were used to prepare new 10- μ l drops. Soybean trypsin inhibitor (1 μ g in 1 μ l medium) was added to some of these drops, while the rest received an equal volume of medium only. Fresh eggs were then added and after incubation for 60 min, these eggs were washed, exposed to capacitated spermatozoa for 90 min and scored for sperm penetration. From Table 4, it is clear that the spermatozoa liberated a substance from the vitelli which blocked sperm penetration and was inhibited by soybean trypsin inhibitor.

DISCUSSION

These experiments indicate that the zona reaction is produced by a trypsin-like

protease liberated from the cortical granules. The protease presumably attacks the species-specific receptor-for-spermatozoa on the zona pellucida. This receptor was earlier shown to be sensitive to very low concentrations of trypsin (Hartmann & Gwatkin, 1971). The fact that, following the zona reaction, the zona pellucida of the hamster egg shrinks markedly on fixation with glutaraldehyde (R. B. L. Gwatkin, unpublished observation) and that the time required for dissolution of the mouse zona pellucida by proteolytic enzymes (Smithberg, 1953), and by crude β -glucuronidase (Gwatkin, 1964) suggests some component of the zona pellucida is removed during the reaction.

Conrad, Buckley & Stambaugh (1971) have proposed an alternative hypothesis, namely that the block to polyspermy is due to a direct effect of a hypothetical cortical granule inhibitor on the trypsin-like protease of the spermatozoa, but they have not presented any supporting evidence to justify their concept. Their demonstration that the zona pellucida of the rabbit egg is more resistant to trypsin after fertilization is not relevant, since there is no zona reaction in this species. Their hypothesis also appears unlikely in view of the known effect of cortical granule products on the zona pellucida and the evidence presented here for a cortical granule protease. Furthermore, such an inhibitor, if it existed, would be working at cross-purposes, facilitating and blocking sperm entry simultaneously; an unlikely possibility.

Our exploration of electrical stimulation for the rupture of cortical granules was based on the report of Tarkowski, Witkowska & Nowicka (1970), which showed that electrical stimulation of the mouse oviduct would cause parthenogenesis of the eggs within it. We supposed that such activation of the eggs might also lead to cortical granule breakdown, which in fact proved to be the case, though the mechanism remains unknown. Experiments are in progress to determine whether heat or depolarization are involved.

Further study is needed to define the time between sperm entry into the egg and the zona reaction. In mating experiments with rats, Braden *et al.* (1954) estimated this time to lie somewhere between 10 min and 2 hr. From their observations on hamster ova, Barros & Yanagimachi (1971) estimated that the zona reaction develops less than 15 min after cortical granule breakdown. Our studies show that approximately 8 min of this time is needed for the protease released from these granules to render the zona pellucida impenetrable to spermatozoa.

Note added in proof

A trypsin-like protease was recently found in the cortical granules of sea urchin eggs and the suggestion was made that it may be involved in the elevation of the fertilization membrane (Vacquier, Epel & Douglas, 1972, *Nature, Lond.* **237**, 34).

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