Properties of the zona pellucida of cytochalasin B-induced triploid mouse eggs

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Summary. The hardening of the zona pellucida as a result of the cortical reaction was estimated in triploid oocytes produced with cytochalasin B by two different procedures. Triploid oocytes resulting from fertilization in the presence of cytochalasin B did not reveal hardening of the zona pellucida unlike triploids obtained by inhibiting in-vitro extrusion of the second polar body in oocytes fertilized *in vivo*.

Introduction

Release of the cortical granule contents initiates the changes in the properties of the zona pellucida known as the zona reaction. After the zona reaction, the zona pellucida becomes impenetrable to spermatozoa and resistant to the action of many physical and chemical agents and to digestion with proteolytic enzymes (see Gulyas & Schmell, 1980).

Cytochalasin B affects microfilaments and fusion membranes (Godman & Miranda, 1978). I have therefore examined the cortical reaction in cytochalasin B-induced triploid mouse oocytes.

Materials and Methods

Experiments were carried out on the eggs of Swiss albino mice fertilized by spermatozoa from F₁ (CBA/H × C57BL) males. The zona reaction of the eggs was estimated by the time taken to digest the zona pellucida enzymically. The eggs were put into 0.1 ml drops of 0.035% pronase (Sigma, St Louis, MO, U.S.A.) in phosphate-buffered saline (pH 7.2) under liquid paraffin at 37°C. Each group of eggs was observed under a dissecting microscope at 5-min intervals and the number of eggs without a zona was counted. The low concentration of pronase leads to a longer time for lysis and so permits precise timing of zona digestion.

In Exp. 1 eggs were released from oviducts of spontaneously ovulating mice at 3 and 3.5 h after the vaginal plug was found and put in drops of medium with 5 µg cytochalasin B/ml under paraffin oil (Niemierko, 1975). After incubation for 7 h they were cleaned of follicular cells and segregated into triploids (3N), diploids (2N) and unfertilized, and placed in pronase solution. Control for the experiment comprised diploid eggs released from females 10 and 10.5 h after the vaginal plug was found.

In Exp. 2 eggs were fertilized *in vitro* in the presence of 5 µg cytochalasin B/ml (Niemierko & Komar, 1976). After incubation for 7 h the eggs were segregated as in Exp. 1 and put into pronase solution. Control groups comprised diploid eggs resulting from fertilization without cytochalasin B and fertilization in the presence of 0.002% DMSO.

In Exp. 3 the time of zona dissolution was monitored and the percentage of hardening in eggs fertilized *in vivo* and *in vitro* was calculated. Pronase was applied to fertilized eggs at 1-h intervals between 1 and 10 h after insemination *in vitro* and between 2.5 and 10 h after mating

The percentage hardening of the zona (%H) was calculated as $(T_m - T_u)/(T_f - T_u) \times 100$, where T_m = mean time of zona lysis in fertilized eggs obtained at 10 h; T_f = mean time of zona lysis in fertilized eggs obtained every 1 h between 1 and 10 h; and T_u = mean time of lysis of unfertilized eggs.

The results were statistically analysed by Student's *t* test.

Results

The results of the zona lysis test are given in Table 1. The time required for zona lysis of triploid and diploid eggs in Exp. 1 was similar to that of control diploid eggs.

Table 1. Times taken to lyse the zona pellucida of mouse eggs

Exp.	Treatment	Ploidy of eggs	No. of eggs (no. of batches)	Mean $\pm$ s.e.m. time of zona lysis (min)
1	Cytochalasin B	3N	87 (6)	72.5 $\pm$ 1.5
		2N	71 (6)	73 $\pm$ 1.1
	None	Unfertilized	139 (6)	51 $\pm$ 1.7
		2N	52 (6)	73.1 $\pm$ 1.4
2	Cytochalasin B	3N	334 (5)	51.5 $\pm$ 1.3
		2N	72 (5)	51.8 $\pm$ 1.7
		Unfertilized	171 (5)	52.0 $\pm$ 1.3
	DMSO	2N	120 (5)	73.0 $\pm$ 1.7
		Unfertilized	192 (5)	51.7 $\pm$ 1.5
	None	2N	112 (5)	72.5 $\pm$ 0.5
		Unfertilized	120 (5)	52.5 $\pm$ 1.2

In the presence of cytochalasin B the zona did not harden and the time of zona lysis between fertilized eggs from Exp. 1 and eggs fertilized and unfertilized in the presence of cytochalasin B from Exp. 2 was significantly different ($P < 0.001$). DMSO had no effect on zona lysis time (Table 1).

The time sequence analysis of zona hardening in the eggs fertilized *in vivo* and *in vitro* is given in Table 2. *In vivo*, hardening of the zona was initiated about 2.5 h after mating, i.e. after the first

Table 2. Timing of hardening (see text) of zona pellucida in oocytes fertilized *in vivo* and *in vitro*

Time after insemination (h)	<i>In vivo</i>			<i>In vitro</i>		
	%H	% fertilized eggs with given %H	No. of fertilized eggs assessed	%H	% fertilized eggs with given %H	No. of fertilized eggs assessed
1	—	—	—	35	5	140
2	—	—	—	35	70	150
2.5	5	2.5	200	—	—	—
3	25	81.5	152	50	86	93
3.5	25	80	145	—	—	—
4	50	80	150	100	83.8	105
5	100	93	115	100	92	150
7	100	93.1	76	100	99	81
10	100	100	130	100	100	70

spermatozoa have penetrated the oocytes. Between 3 and 3.5 h as many as 80% of eggs showed 25% hardening of the zona, indicating that at the time of transfer to the medium with cytochalasin B in Exp. 1 most oocytes had completed the cortical reaction. However, in-vitro-initiated zona hardening continued in the presence of cytochalasin B because the mean time of zona lysis in 3N and 2N eggs from Exp. 1 was not shorter than the time of 100% hardening of the zona in Exp. 3.

The yield of fertilized eggs increased from 5 to 70% between 1 and 2 h after insemination *in vitro*, but only a few of the eggs indicated hardening of the zona by the first hour. Hardening of the zona in oocytes fertilized *in vivo* and *in vitro* developed similarly and 100% hardening of the zona occurred at 5 and 4 h respectively after fertilization.

Discussion

Cytochalasin B-induced triploid mouse oocytes obtained by two means differed in the properties of their zona pellucida. Triploids resulting from fertilization in the presence of cytochalasin B did not reveal hardening of the zona pellucida, although the zonae of eggs fertilized *in vivo* and then treated with cytochalasin B continued to harden. The temporal relationship between sperm entry and cortical granule release (Inoue & Wolf, 1975; Nicosia, Wolf & Inoue, 1977; Fukuda & Chang, 1978; Sato, 1979) and the timing of hardening of the zona (present experiment) provide evidence that triploid eggs obtained by treating in-vivo fertilized eggs with cytochalasin B undergo the cortical reaction and restructuring of microfilaments in the egg cortex before the cytochalasin can act.

It seems probable that cytochalasin B used to block cytokinesis of the second maturation division prevented fully or partly the extrusion of cortical granules when present during fertilization. Cytochalasin B causes corrugation of the oocyte surface (Johnson, Eager, Muggleton-Harris & Grave, 1975) which might prevent the approximation of cortical granules to the membrane and in this way interfere with fusion.

The action of cytochalasin B disrupts some elements of the cytoskeletal system (Godman & Miranda, 1978) which are involved in the process of exocytosis of cortical granules (Szöllösi, 1967; Gulyas, 1980). Wassarman, Ukena, Josefowicz, Letourneau & Karnovsky (1977) have shown that unfertilized mouse oocytes do not undergo a cortical reaction although pseudocleavage is induced by cytochalasin B. It is also probable that oocytes fertilized in the presence of cytochalasin B undergo a partial cortical reaction as during premature granule release which does not affect egg penetrability (Nicosia *et al.*, 1977) because there is too low a concentration of cortical enzymes to have a significant effect on the properties of the zona.

The hardening of the zona pellucida is a time-dependent process and does not differ *in vivo* or *in vitro*, being 2.5 h *in vivo* (2.5–5 h) and 3 h *in vitro* (1–4 h). In experiments with calcium ionophore A23187 (Schmell & Gulyas, 1980) zona hardening occurred between 2 and 3 h after exposure to ionophore and at 3 h when exogenous peroxidase was added to inactivate eggs.

The ovoperoxidases from cortical granules modify the sperm receptor, a glycoprotein on the zona designated ZP3 (Bleil & Wassarman, 1980) and also by cross-linking of tyrosine residues, modified glycoprotein ZP2, which provides hardening of the zona (Schmell & Gulyas, 1980; Bleil, Beall & Wassarman, 1981).

The present results and those of earlier work (Niemierko & Komar, 1976) indicate that these functions are not expressed in the presence of cytochalasin B and makes the supposition that cytochalasin B interferes with cortical granule extrusion more probable.

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