Action of phorbol myristate acetate (PMA) at fertilization of mouse oocytes *in vitro*

ANNA NIEMIERKO AND ALDONA KOMAR

Laboratory of Experimental Embryology, Department of Histology and Embryology, Medical School of Warsaw, Chałubińskiego 5, 02-004 Warsaw, Poland

SUMMARY

Phorbol ester (PMA) in concentration 5 and $10 \, ng \, ml^{-1}$ blocks cytokinesis of the second maturation division in mouse oocytes. Karyokinesis is not impaired and digynic triploid oocytes are obtained which undergo first cleavage division.

Effectiveness of blocking cytokinesis is dependent on the timing of exposure of oocytes to PMA action. When oocytes are subjected to PMA at the onset of the second maturation division only 14·5 % of eggs are triploid. PMA present during fertilization *in vitro* (about 1 h exposure to PMA) induces triploidy in 40 % eggs. Extending the time of exposure of oocytes to 2 h produces 76 % tripronucleate eggs.

Applicability of PMA is compared with the use of cytochalasin B to induce triploidy in the mouse.

INTRODUCTION

Cytochalasin B, the drug affecting microfilaments (Godman & Miranda, 1978), blocks cytokinesis of the second maturation division when present during fertilization of mouse oocytes (Niemierko, 1975; Niemierko & Komar, 1976).

There are reports that phorbol myristate acetate (PMA) blocks cytokinesis without impairing karyokinesis as well, which results in binucleation of sea urchin (Bresh & Arendt, 1978), frog (Ellinger, 1982) and mouse (Sawicki & Mystkowska, 1981) blastomeres.

The aim of the present work was the application of PMA to block cytokinesis of the second maturation division in mouse oocytes fertilized *in vitro*.

MATERIALS AND METHODS

Fertilization *in vitro*

Oocytes were inseminated according to the method of Toyoda, Yokoyama & Hosi (1971) as described in detail on our earlier work (Niemierko & Komar, 1976). The oocytes were obtained from outbred Swiss albino females induced to superovulate by *i.p.* injections of 5 i.u. PMSG (Gestyl, Organon) and hCG (Chorulon, Intervet) given 43–48 h apart. Females were killed 13 h after the second injection. Sperm was collected from F_1 (CBA/H×C57B1) males.

Chemicals

Phorbol 12-myristate 13-acetate (PMA; Sigma, lot no. 99c-0131) was used in concentrations $5 \, \text{ng ml}^{-1}$ and $10 \, \text{ng ml}^{-1}$, which were obtained by dissolving stock solution containing $0.1 \, \text{mg}$ PMA per 1 ml DMSO in fertilization medium.

Three experiments were carried out.

Experiment I

$1-1.5 \, \text{h}$ after mixing the gametes, oocytes were treated with $0.25 \, \%$ hyaluronidase in $0.9 \, \%$ NaCl to remove the cumulus oophorus. Oocytes showing the presence of cytoplasmic bulge heralding the second polar body and freshly penetrated oocytes with a spermatozoon under the zona pellucida were placed in a drop of medium containing $5 \, \text{ng ml}^{-1}$ PMA under paraffin oil. After 1 h of observation under dissecting microscope (warm stage), the eggs were cultured for $3-4 \, \text{h}$ in $37 \, ^{\circ}\text{C}$ and $5 \, \%$ CO_2 in air.

Experiment II

The eggs were fertilized *in vitro* in the presence of $5 \, \text{ng ml}^{-1}$ or $10 \, \text{ng ml}^{-1}$ PMA. Control eggs were fertilized in PMA-free medium. All eggs were incubated for 6 h. In order to determine whether PMA-induced triploids cleaved to 2-cell stage, a few tripronucleate eggs from this experiment were cultured in the medium without PMA for 20 h after mixing the gametes.

Experiment III

Oocytes were preincubated for $1.5 \, \text{h}$ in fertilization medium containing $5 \, \text{ng ml}^{-1}$ PMA before they were fertilized *in vitro* in the presence of $5 \, \text{ng ml}^{-1}$ PMA. The double control experiments were carried out. In Control 1 eggs preincubated $1.5 \, \text{h}$ *in vitro* were fertilized. In Control 2 eggs obtained from females $14.5 \, \text{h}$ post hCG were fertilized. Each experiment lasted 5 h and then the eggs were fixed. Whole-mount preparations were made from all the eggs according to Tarkowski & Wróblewska (1967). Eggs were examined with light microscope. The results were treated statistically with χ^2 test.

RESULTS

PMA at a concentration 5 and $10 \, \text{ng ml}^{-1}$ prevents cytokinesis of the second maturation division of the mouse oocytes, so that digynic triploids arise (Figs 1, 2). When oocytes were subjected to PMA at the onset of the second maturation division, yield of triploids, reached only $14.4 \, \%$ (Table 1). The eggs used in this experiment were at different stages of the second maturation division, which correlated with the size of the cytoplasmic bulge heralding the second polar body. PMA did not cause retraction of this bulge, the nascent polar body. Cytokinesis was effectively blocked only in freshly penetrated eggs. In such eggs the spindle of metaphase II has not rotated.

The percent of triploid eggs increased to about 40 % in Experiment II (Table 2), when oocytes were fertilized in the presence of PMA. Because spermatozoa penetrated the oocytes at the end of the first hour, PMA acted for about one hour on unfertilized oocytes. Neither concentration of PMA impaired the fertilization process (in control eggs the rate of fertilization was insignificantly higher). However, at both concentrations the yield of triploids was similar. As shown in
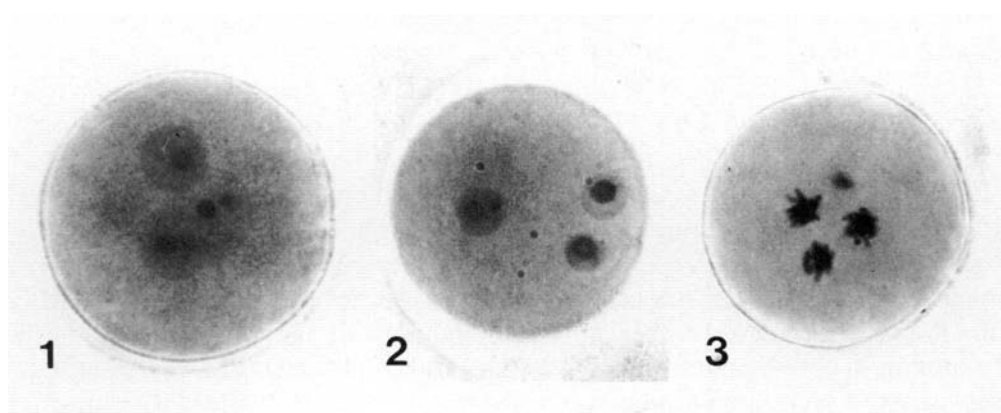
Fig. 1. Triploid mouse oocyte obtained with $5 \, ng \, ml^{-1}$ PMA. Three pronuclei are seen. ×600.

Fig. 2. Another triploid oocyte obtained with the same technique. ×700.

Fig. 3. The first cleavage division of triploid oocyte. Three groups of chromosomes are seen. In the neighbourhood of the chromosomes an artefact is visible. ×600.

Table 1. *Blocking of second polar body extrusion by 5 ng ml^{-1} PMA*
(Experiment I)

Ploidy of eggs	No. of eggs (%)	
3n	9	(14·5)
2n+2nd p.b.	53	(85·5)
Total	62	(100·0)

Table 2. *Fertilization of mouse oocytes* in vitro *in the presence of PMA*
(Experiment II)

Ploidy of eggs	PMA						Control	
	5 ng			10 ng			Control	
	No. of eggs (%)			No. of eggs (%)			No. of eggs (%)	
3n	70	(39·3)		19	(39·6)		1	(0·6)
4n	8	(4·5)		2	(3·6)		2	(1·2)
3n+2nd p.b.	12	(6·7)		4	(7·3)		21	(12·5)
4n+2nd p.b.	3	(1·7)		—	—		—	—
2n+2nd p.b.	85	(47·8)		30	(54·5)		144	(85·7)
Total	178	(100·0)	(74·8)	55	(100·0)	(75·4)	168	(100·0)	(81·6)
Activated	3*		(1·3)	8†		(10·9)	5‡		(2·4)
Unfertilized	57		(23·9)	10		(13·7)	33		(16·0)
Grand total	238		(100·0)	73		(100·0)	206		(100·0)

*3×2 pronuclei
†7×2 pronuclei; 1×1 pronucleus+2nd p.b.
‡5×1 pronucleus+2nd p.b.

Table 3 half of triploids obtained with PMA enters (Fig. 3) or finishes the first cleavage division.

The incidence of triploidy was highest (72 %) in Experiment III (Table 4), when oocytes, preincubated with PMA, were fertilized in the presence of $5\,\mathrm{ng\,ml^{-1}}$ PMA. However, preincubation *in vitro* decreased the rate of fertilization itself (compare Control 1 and Control 2). In Experiment III PMA acted for $1\cdot5\,\mathrm{h}$ on untreated eggs (pre-incubation) and about $1\,\mathrm{h}$ on unfertilized eggs (as in Experiment II). Since PMA is ineffective after cytokinesis starts (cf. Exp. I), it might be suggested that the high yield of triploidy achieved in Experiment III is due to prolongation of PMA treatment to about $2\,\mathrm{h}$.

Among oocytes spontaneously activated in the presence PMA, 2-pronuclear eggs without second polar body preveil. Because up to $20\,\mathrm{h}$ after HCG injection the majority of activated eggs remains haploid with second polar body (Kaufman & Surani, 1974, controls to experiments II and III), it is concluded that diploid, two pronuclear, eggs are the result of PMA action.

The rate of polyspermic (3n+2nd p.b; 4n; 4n+2nd p.b.; 5n) eggs in Experiment II and III was similar to that in the Control. The majority of the polyspermic eggs

Table 3. *First cleavage division of triploid eggs obtained with PMA in Experiment II*

Stage of embryos	PMA No. of eggs (%)		Control No. of eggs (%)	
2-cell	3	(13·0)	12	(7·7)
mitosis of I c.d.	9	(39·1)	12	(92·3)
3n	11	(47·9)	—	—
Total	23	(100·0)	13	(100·0)

Table 4. *Fertilization in vitro of the mouse oocytes preincubated for $1\cdot5\,h$ with $5\,ng\,ml^{-1}$ PMA (Experiment III)*

Ploidy of eggs	PMA No. of eggs (%)			Control 1 No. of eggs (%)			Control 2 No. of eggs (%)		
3n	36	(72·0)		—	—		—	—	
4n	2	(4·0)		—	—		—	—	
n	1	(2·0)		—	—		—	—	
3n+2nd p.b.	3	(6·0)		11	(20·4)		12	(11·8)	
2n+2nd p.b.	8	(16·0)		43	(79·6)		90	(88·2)	
Total	50	(100·0)	(54·9)	54	(100·0)	(65·0)	102	(100·0)	(81·6)
Activated	17*		(18·7)	8†		(9·7)	—		—
Unfertilized	24		(26·4)	20		(24·4)	23		(18·4)
Grand total	91		(100·0)	82		(100·0)	125		(100·0)

* 5×1 pronucleus without 2nd p.b.; 10×2 pronucleus; 2×1 pronucleus+2nd p.b.
† 8×1 pronucleus+2nd p.b.

were dispermic. From these results it would appear that PMA does not affect the block to polyspermy at the plasmalemma level.

DISCUSSION

Phorbol ester (PMA) prevents cytokinesis of the second maturation division of the mouse oocytes. The action of PMA is most effective when it precedes cytokinesis for about two hours. Karyokinesis of the second maturation division was not impaired and, as in the presence of cytochalasin B, digynic triploid oocytes were obtained. In contrast to the rapidly acting cytochalasin B, which caused retraction of large bulges of cytoplasm heralding the second polar body (Niemierko, 1975; Niemierko & Komar, 1976), PMA did not inhibit cytokinesis of the second maturation division once it had started. PMA does not inhibit initiated cytokinesis of the first cleavage division of mouse blastomeres and immediate cleavage of spontaneously activated eggs (Niemierko & Komar, unpublished data).

Induction of triploidy with PMA is procedurally more complicated, but it is as effective as the method using cytochalasin B. Half of triploids obtained with the application of either drug undergoes first cleavage division.

The mechanism of blocking cytokinesis by PMA is unrecognized. PMA causes many cellular effects. Binding of PMA to a receptor, protein kinase C, which is the integral enzyme of cell membrane, mediates rapid changes in membrane structure (Kraft & Anderson, 1983; Parker, Stabel & Whaterfield, 1984). Scarce study on effects of PMA on the cytoskeleton of differentiated cells suggest selective action of the drug on intermediate filaments. PMA induces translocation of these filaments and the redistribution of cytoplasmic action and microtubules (Croop, Toyama, Długosz & Holtzer, 1980; Phaire-Washington, Silverstein & Wang, 1980).

The cytoskeleton of unfertilized eggs and one-cell zygotes comprises tubulin, actin and α-actinin (Wassarman & Fujiwara, 1978; Lehtonen & Badley, 1980). Intermediate filaments appear as non-filamentous cytokeratin and scarce cyto-keratin filaments appear until cleavage-stage embryos (Lehtonen et al. 1983). Unfortunately, myosin in mouse oocytes and one-cell zygotes has not been investigated, although it is one of the major components of the embryo cyto-skeleton already in 2-cell stage (Sobel, 1983). Recently, studies have been made on the organization and dynamics of actin microfilaments (Maro, Johnson, Pickering & Flach, 1984) and microtubules (Schatten, Simerly & Schatten, 1985) during fertilization events. Despite extensive studies of the cytoskeleton of early mouse embryos, little is known at present about the functional cooperation of cytoskeletal elements, or about the interaction of the cytoskeleton with plasma membrane.

It seems probable that PMA blocks cytokinesis of the second maturation division indirectly. Because karyokinesis of this division is not disturbed by PMA this excludes the action of the drug on the spindle microtubules. The present study

reveals that PMA does not interfere with organized and functioning actin filaments during cytokinesis. Maybe PMA affects precytokinetic organization of actin and/or the connections of this cytoskeletal system with plasma membrane. An important initial event during extrusion of second polar body is the attachment of the equator of the meiotic spindle to the membrane. Thus the connection of the actin equatorial part of the meiotic spindle with the overlying egg plasma membrane could be a target site for PMA action.

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