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Original Article

Parthenogenetic activation of mouse oocytes using calcium ionophores and protein kinase C stimulators

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ABSTRACT Fertilization involves the production of inositol trisphosphate and diacylglycerol with a subsequent increase in intracellular calcium concentration ($[Ca^{2+}]_i$) and the activation of a calcium-dependent protein kinase, the so-called protein kinase C (PKC). Methods of parthenogenetic activation have focused on this calcium wave which seems to be large enough to generate all the responses associated with fertilization and even finally inducing the activation of PKC activity. The specific stimulation of PKC by phorbol esters in turn elicits $[Ca^{2+}]_i$ oscillations although no reports exist claiming that the mere activation of this protein is capable of sustaining embryonic development. In this paper we describe the effect of different calcium ionophores and phorbol esters as parthenogenetic agents on mouse oocytes compared with ethanol as the standard procedure. Phorbol esters (OAG) fail to activate a significant number of oocytes, with very few reaching blastocyst stage. However, when a calcium ionophore (A23187) is added, the percentage of embryos reaching the blastocyst stage increases to such an extent that it is the best chemical method assayed to date. We conclude that incubation with both compounds combined inhibits feed-back processes between the above reactions and so induces a more physiologic parthenogenetic activation.

KEY WORDS: *mammalian oocyte activation, Ca^{2+} ionophores, PKC*

Introduction

The activation of oocyte metabolism during the process of fertilization is shown through a series of responses that are usually classified as "early" or "late" according to the moment they appear. The former are characterized by the exocytosis of cortical granules (cortical reaction), involving modifications in the glycoproteins of the zona pellucida destined to block polyspermia. The late reactions include the restart of meiosis (stopped at the metaphase of the second meiotic division), the emission of the second polar body, the formation and migration of pronuclei, the initiation of maternal mRNA translation and the synthesis of new DNA (Nuccitelli, 1991).

All these reactions originate due to a repetitive and transient rise in the intracellular concentration of calcium ($[Ca^{2+}]_i$) (Cuthbertson *et al.*, 1981; Sagata *et al.*, 1989; Kline and Kline, 1992; Xu *et al.*, 1994). Several hypotheses exist concerning the way this signal starts and propagates (Jaffe, 1989; Shen, 1989; Nuccitelli, 1991; Bement, 1992). However, it is considered that sperm ligands may initiate mammalian egg activation by stimulating phosphoinositide metabolism via protein G, which results in the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Miyazaki, 1988; Jaffe, 1989; Moore *et al.*, 1993; Moore *et al.*, 1994). Alternatively, these second messengers could also be induced by some kind of factor released into the cytoplasm by the sperm (Swann, 1994). IP_3 is a second messenger which acts by releasing calcium from intracellular stores. This first Ca^{2+} release would then stimulate subsequent calcium transients, although evidence concerning the molecular mechanism is still unclear (Miyazaki, 1988; Swann, 1992; Shiina *et al.*, 1993; Kline and Kline, 1994). DAG activates the protein kinase C (PKC), a calcium-, phospholipid-dependent protein kinase which has been implicated in the various processes resulting from fertilization, from the slight alkalinization of the cytoplasm (Shen, 1989) to the synthesis, protein phosphorylation and modification of the zona pellucida preventing polyspermia (Endo *et al.*, 1987; Colonna *et al.*, 1989).

The different methods of parthenogenetic activation used to date attempt to induce these reactions directly or indirectly. In fact, eggs can be activated by agents that induce a rise in the intracellular concentration of calcium ($[Ca^{2+}]_i$), either by enabling the entrance of this cation from the external medium (as when incubating in dilute solutions of ethanol) (Cuthbertson, 1983; Kaufman, 1983; Onodera and Tsunoda, 1989) or by the

Abbreviations used in this paper: PMA, 4β-phorbol 12-myristate 13-acetate; PKC, protein kinase C; OAG, 1-oleyl-2-acetylglycerol; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PVP, polyvinylpyrrolidone.

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action of specific ionophores that liberate calcium from intracellular stores (Steinhardt *et al.*, 1974; Vincent *et al.*, 1992). On the other hand, the use of phorbol esters as activators of the PKC elicits sustained $[Ca^{2+}]_i$ oscillations, as occurs in a normal fertilization (Cuthbertson and Cobbold, 1985). However, it has not been described that in mice the effects of PKC activation alone are sufficient to induce the proper completion of meiosis and the entrance of the embryos into the mitotic cycles characteristic of the cleavage stage. It has even been claimed that PKC stimulation could inhibit cell division (Mystkowska and Sawicki, 1987).

In this paper we have compared the effect of several calcium ionophores and phorbol esters acting as inductive agents of parthenogenetic activation, either alone or in combination. Activation was considered successful according to the percentage of eggs reaching the 2-cell or blastocyst stage, since the mere emission of the second polar body and formation of pronuclei cannot be considered as a guarantee of subsequent sustained development (Kaufman, 1983; Gallicano *et al.*, 1993).

Results

Treatment with calcium ionophores and phorbol esters separately

Table 1 shows the results from the exposition to 1 and 5 µM ionomycin or calcimycin (A23187) for 5 to 10 min. These data indicate that oocytes are very sensitive to treatment with ionomycin, since 1 µM has a scarce activating effect while 5 µM is too aggressive. Specifically, the embryo lethality rate was 50% to 60% 24 h after treatment and there were a great number of non-viable embryos characterized by their multiple cytoplasmic fragmentations. On the other hand, A23187 induces only slight activation at a concentration of 1 µM, but incubation with 5 µM of this compound for 5 min yields a rate of 56% reaching the 2-cell stage, and 40% when the treatment is lengthened to 10 min. It is worth mentioning that these percentages correspond to embryos that have not been exposed to cytochalasin D and are, therefore, haploids. These embryos have lower viability (Kaufman, 1983), with many cell divisions which end in fragmentations of the blastomeres. This result shows, however, that both the cellular fragmentations and the death rate 24 h after exposure to the activating agents are higher with ionomycin than with A23187.

TABLE 1

EMBRYO DEVELOPMENT 24 H AFTER TREATMENT WITH DIFFERENT IONOMYCIN AND/OR A23187 CONCENTRATIONS AND EXPOSURE TIMES

		Ionomycin		A23187	
		1 µM	5 µM	1 µM	5 µM
5 min	2 cells	21.4	-	-	56
	Frag[a]	7.1	30	-	11.1
	Death		50	-	-
10 min	2 cells	14.2	-	18.8	40
	Frag.	21.4	10	9.9	26.6
	Death	-	60	-	-

Data in %. [a]Embryos with fragmented blastomeres.

TABLE 2

EMBRYO DEVELOPMENT 24 H AFTER TREATMENT WITH DIFFERENT OAG AND/OR PMA CONCENTRATIONS AND EXPOSURE TIMES

		PMA		
		10^{-5}M	10^{-6}M	10^{-7}M
5 min	2 cells	-	-	-
	Death	-	33.3	25
15 min	2 cells	-	-	-
	Death	-	37.5	22.2
		OAG		
		10^{-3}M	10^{-4}M	10^{-5}M
5 min.	2 cells	37.5	12.5	50
	Death	-	-	-
15 min	2 cells	22.2	44.4	55.5
	Death	-	-	-

Data in %.

Regarding PKC activators, we assayed 10^{-5}, 10^{-4} and 10^{-3} M 1-oleyl-2-acetylglycerol (OAG) and 10^{-7}, 10^{-6} and 10^{-5} M 4β-phorbol 12-myristate 13-acetate (PMA) for 5 to 15 min (Colonna *et al.*, 1989) (Table 2). It was observed that PMA cannot induce the activation and development of an appreciable number of oocytes but that it does cause some cell death which could be due to the effect of maintaining the non-activated oocytes *in vitro*. On the other hand, OAG induces a certain activation at the three concentrations assayed after both 5 and 15 min of incubation.

Combined treatment

We considered that the specific simultaneous induction of different metabolic pathways (calcium and PKC) could result in an improvement of the final activation yield. For this reason, and in the light of the above results, we started testing the combined activating potential of 5 µM A23187 and 10^{-4} M OAG for 5 and 10 min. Two concentrations with different incubation times were tested: a) A23187 for 10 min followed by 3 short washings in MA-1 and 5 min in OAG and b) OAG for 5 min followed by 5 min in A23187. In both cases the activation induced was similar, with approximately 45% of the oocytes reaching the 2-cell stage after 24 h in culture (not shown). It can be inferred from these data that there does not seem to be any effect on activation due to time differences between the induction of the reactions responsible for raising the Ca^{2+}_i concentration and those that activate the PKC.

In order to study whether both processes could act simultaneously inducing stronger activations, a medium containing 5 µM A23187 and 10^{-4} M OAG was tested for different periods of time. In these circumstances, the rate of embryos reaching the 2-cell stage hardly changed with any of the 5 incubation times used (8, 12, 16, 20 and 24 min), with 77.8% of diploid oocytes reaching this stage within 24 h of activation. This is why, in order to appreciate differences in the incubation times, we cultured the embryos for 5 days, measuring the percentage reaching the blastocyst stage (Fig. 1). These data show that 5 min of incubation produces enough parthenogenetic activation to induce a good rate of development to the blastocyst stage (54.6%).

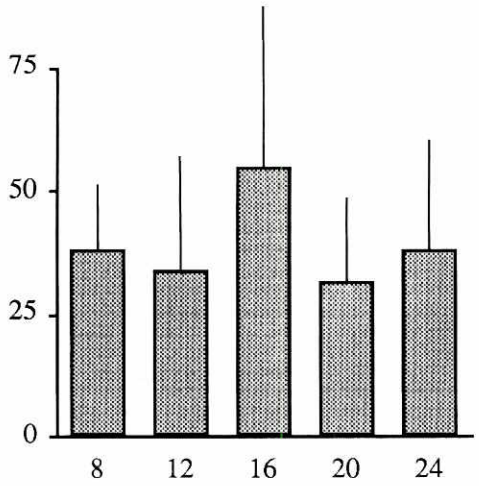

Fig. 1. Percentage of blastocyst development after combined A23187+OAG activation. *Mean and SD of 4 measures. n= 525.*

Accordingly, the following treatments were chosen as the most efficient:

- calcium ionophore: 5 µM A23187 for 5 min.
- PKC stimulator: 10^{-4} M OAG for 10 min.
- consecutively: 5 µM A23187 for 10 min followed by 5 min in 10^{-4} M OAG.
- jointly: 5 µM A23187 and 10^{-4} M OAG for 15 min.

These treatments were applied to the oocytes, which were afterwards exposed to cytochalasin D. The development potential of these oocytes compared to the ones exposed to the traditional method of ethanol activation was then analyzed. They were also compared to the non-activated controls (Table 3). This table shows that activation induced only by OAG does not entail major improvement over the control diploid embryos. On the other hand, activation with ethanol yields rates of attainment of the different embryonic stages similar to those achieved using A23187 and OAG consecutively. However, these activation rates improve in oocytes that have been exposed only to the calcium ionophore (group 4). The improvement is even greater when the activation is induced by simultaneous exposure to A23187 and OAG. This latter treatment ameliorates the embryo's developmental capacity in each and every stage considered, thus confirming that the combined action of both chemicals results in more physiological activation conditions. This is something that can also be appreciated comparing the cell number reached by these embryos. Table 4 shows the number of cells of embryos activated with ethanol vs. A23187+OAG after 102 h of *in vitro* culture. Significant differences exists in both groups, with ethanol-activated embryos showing a smaller cell proliferation rate than the ones activated with the other method.

Discussion

The biochemical mechanism that is activated by fertilization and provokes the inhibition of polyspermia and the renewal of meiosis is complex and not completely understood in mammals. The different parthenogenetic activation methods make it possible to study these reactions, since they attempt to reproduce (at least, partially) their effects. Traditionally, however, these methods have focused on aspects such as the elevation of $[Ca^{2+}]_i$ that cannot explain the phenomenon on their own. For this reason we have studied the effect that the stimulation of several metabolic pathways has on inducing parthenogenetic activation and, more important, permitting sustained growth during the preimplantation period.

First of all, we tested ionomycin and A23187, two ionophores that induce an increase in the intracellular calcium concentration. No appreciable activation was observed in oocytes treated with the former, so it can be assumed that the calcium rise caused by ionomycin is not sufficient. According to Colonna *et al.* (1989) the exposure of oocytes to 1 µM inomycin yields a 1 µM increase in $[Ca^{2+}]_i$. This increase is five times smaller that the one obtained when oocytes are activated with ethanol (Cuthbertson *et al.*, 1981). On the other hand, the A23187 induces significant parthenogenetic activation yielding high rates of development to the blastocyst stage. The differences between 1 µM and 5 µM have been explained as follows: low ionophore concentrations do not allow a high enough rise in the $[Ca^{2+}]_i$ to allow the cells to enter anaphase II. The oocytes actually start meiosis again, emit the second polar body and enter a new metaphase (metaphase III) where they are finally halted (Vincent *et al.*, 1992).

The second group of compounds tested were the phorbol esters. These are diacylglycerol analogues and therefore are capable of activating PKC. Traditionally, PKC has been considered to be implicated in the regulation of the Na^+/H^+ pump responsible for regulating the pH of the cytoplasm and for the "late" reactions of fertilization (Jaffe, 1989; Shen, 1989). However, it is also thought that it can take part in the quick blockage of polyspermia by modifying the zona pellucida (Endo *et al.*, 1987). Colonna *et al.* (1989) observe that treatments of 10^{-7} M PMA or $2x10^{-4}$ M OAG for 15 min induce the formation of pronuclei in 77% of the anisogenic mice treated. In fact, the action

TABLE 3

PARTHENOGENETIC EMBRYO DEVELOPMENT AFTER ACTIVATION WITH DIFFERENT METHODS

Treatments	1 n=264	2 n=261	3 n=252	4 n=245	5 n=277	6 n=277	7 n=340
2-Cell	55.1*	42.7*	74.3	76.2	55.6*	76.4	84.4
SD	(7.6)	(4.8)	(1.5)	(2.4)	(7.3)	(1.9)	(2.4)
p				0.0145			
4-Cell	27.7*	29.3*	49.1	50.6	31.1*	49.6	66.6
SD	(2.4)	(3.1)	(2)	(4.5)	(3.3)	(2.7)	(2.6)
p				0.0079			
Morulas	9*	16.8*	37	36.5	21.1*	41.2	55.3
SD	(1.1)	(1.7)	(2.4)	(4.2)	(3.5)	(3.4)	(1.3)
p				0.0002			
Blastocysts	2.3*	12.5*	23.7	32.3	17.7*	25.2	42.1
SD	(1.1)	(3.2)	(2.4)	(2.7)	(3.6)	(1.1)	(1)
p				0.0001			

Data in %. Standard deviation (SD) in brackets. Treatment 1: haploid control; 2: diploid control; 3: 7% ethanol, 5 min; 4: 5 µM A23187, 5 min; 5: 10^{-4} M OAG, 10 min; 6: 5 µM A23187, 10 min followed by 10^{-4} M OAG, 5 min; 7: 5 µM A23187 and 10^{-4} M OAG jointly for 15 min. Data from 6 measures. Significant differences according ANOVA when $p \leq 0.05$. *Groups significantly different from group 7 according to Duncan's test.

mechanism of both chemicals is the same although OAG metabolizes more quickly than PMA, so a higher concentration of the former is needed to achieve the same effect. This different metabolism is also the reason why activation with PMA yields diploid embryos, while OAG provokes the second polar body emission. According to Mystkowska and Sawicki (1987), PKC activators prevent cytokinesis but not kariokinesis when they are applied in the G2/M phase of the cell cycle. The longer stay of non-metabolized PMA in the membrane seems to enhance this inhibitory effect.

TABLE 4

CELL NUMBER OF FERTILIZED AND PARTHENOGENETIC BLASTOCYSTS

	Parthenogenetic ethanol n=13	Parthenogenetic A23187+OAG n=36	Fertilized n=56
Cell number	33.5	47.1	54.5
SD	(4.5)	(6.3)	(7.1)
p		0.00001	

Standard deviation (SD) in brackets. Significant differences when $p \leq 0.05$

In our case, successful activation was considered as depending on the developmental ability of the embryos. OAG proved clearly better than PMA, which is probably due to differences in the capacity of the two compounds to be metabolized or maintained in the egg membrane of OF1 mice. In this regard, it is worth noting that Cuthbertson and Cobbold (1985), using anisogenic MF1 mice, induced a percentage of pronuclei formation similar to that of Colonna *et al.* (1989) although they only report the preimplantation development after PMA treatment in F1 hybrid mice. Using this method they achieved a percentage of embryos developing to morula and blastocyst similar to the rate obtained after ethanol activation. Our data do match theirs, as activation with PKC stimulators (OAG) did not mean an improvement with respect to ethanol (Table 3). However, treatment with PMA was clearly harmful for our oocytes. Strain differences have been previously described concerning this chemical. In fact, PMA induces disassembly of the spindle microtubules and the formation of pronucleus-like structures in the CD-1 strain but not in CF-1 (Moore *et al.*, 1995). In addition, the longer stay of PMA in the membrane avoiding second polar body extrusion provokes the degeneration of oocytes within 1 h after exposure to this compound (Moses and Kline, 1995). This could explain the high rates of death we have observed. It should be finally noted that when phorbol esters are the only activating agent, not only PKC is induced but there is also a calcium release from intracellular stores. This release is less intense than in fertilization or in ethanol activation but it could be important enough to promote the activating effects caused by PMA in some strains (Cuthbertson and Cobbold, 1985).

In the case of activation with A23187 followed by OAG, once again there is no improvement over A23187 alone. The reason may be that the time elapsed between incubation in A23187 and exposure to OAG is long enough to unlink this two reactions. If this were the case, the only significant activating effect would be the one promoted by the A23187. From the activating effects of ethanol, A23187 and OAG, it can be concluded that the increase in $[Ca^{2+}]_i$ seems to be enough to induce all the phenomena associated with activation. It has also been described that calcium stimulates PKC activity (Gallicano *et al.*, 1993) which, in turn, elicits a rise in the concentration of this cation (Cuthbertson and Cobbold, 1985). We consider that combined incubation with A23187 and OAG induces both effects (i.e. calcium rise and PKC activation) simultaneously, making it possible to avoid decreasing the reaction yield due to feed-back processes. In fact, this method has given us the best results concerning not only the ability to promote development throughout the preimplantation period but also to induce higher cell proliferation rates. It is also important to note that the cell number obtained with A23187+OAG is quite similar to the one obtained with fertilized OF1 embryos cultured *in vitro* (Uranga and Arechaga, submitted). This is why we believe that this kind of activation reproduces the mechanisms responsible for the renewed onset of meiosis in a more physiological way than previously described.

Materials and Methods

Murine egg recovery

Random-bred OF1 females (Iffa-Credo, France) of 5 weeks of age were superovulated by intraperitoneal injection at midday of 5 IU pregnant mares' serum gonadotrophin (PMSG) followed 48 h later by 5 IU human chorionic gonadotrophin (hCG). The females were caged with OF1 males and mating was confirmed the following morning by the presence of vaginal plugs; the day of the vaginal plug was considered Day 1 of development. Ovulation and mating were assumed to occur 12 h after hCG administration. Females were sacrificed by cervical dislocation between 18.5 and 19.5 h after hCG injection. Non-fertilized eggs were flushed from the oviduct in MA-1 medium, a T6-derived medium (Howlett *et al.*, 1987) buffered with 20.8 mM HEPES and a smaller concentration of sodium bicarbonate (4.15 mM). Eggs were denuded from cumulus cells with a 0.1% hyaluronidase solution (Hogan *et al.*, 1986).

Parthenogenetic activation

All treatments were made 20 h after hCG administration because this is when the highest yields have been attained under the different protocols assayed to date (Kaufman, 1983; Kubiak, 1989; Vincent *et al.*, 1992).

Activation using ethanol solutions

Oocytes freed from cumulus cells were incubated in 7% ethanol (Merck) in MA-1 medium for 5.5 min. Next, they were washed and cultured in 1μg/ml cytochalasin D (Sigma) for 5 h in T6 to avoid second polar body emission, which would produce diploid parthenogenetic embryos. Finally, oocytes were washed and cultured in T6 medium+BSA (Cuthbertson, 1983).

Activation with calcium ionophores and PKC stimulators

Ionomycin (Molecular Probes) and the ionophore highly sensitive to calcium A23187 (Sigma) were tested at concentrations ranging from 1 to 5 μM. Oocytes were treated for 5 to 10 min at 37°C (Colonna *et al.*, 1989). As PKC activators 10^{-5} to 10^{-3} M 1-oleyl-2-acetylglycerol (OAG, Sigma) and 10^{-7} to 10^{-5} M 4β-phorbol 12-myristate 13-acetate were assayed for 5 to 15 min (Colonna *et al.*, 1989). Incubations were made both separately, and in combination consecutively to the calcium ionophores, washing the oocytes for 15 min before exposure to a new agent. PMA, OAG and A23187 stock solutions were made using dimethylsulfoxide (DMSO) as solvent and stored at -20°C until used. The presence of this solvent in the incubation medium never exceeded 0.1%

(Colonna *et al.*, 1989). Finally, oocytes were treated with cytochalasin D as previously described to obtain diploid embryos.

Culture and cell number analysis of embryos

Following activation, oocytes were cultured in microdrops of T6 medium+BSA (Howlett *et al.*, 1987) supplemented with 0.02 mM EDTA (Abramczuk *et al.*, 1977) and overlaid with mineral oil (Sigma) (Brinster, 1963) in Petri dishes (Falcon, 3001) in a 5% CO_2 incubator at 37°C. A total of 20 oocytes were cultured per drop of 7.5 µl. The percentage of embryos reaching the 2- and 4-cell stages after 24 and 48 h, and the morula and blastocyst stages after 72 and 120 h of culture were quantified under a Leitz M10 microscope.

The cell number of the embryos was calculated after 102 h of *in vitro* development according to a modification of the method described by Johnson and Ziomek (1983). Briefly, zona pellucida was removed after 5 min in 0.5% pronase in MA-1+PVP (Pratt, 1987) and embryos incubated with 50 µg/ml DAPI in MA-1+BSA for 15 min. After washing, embryos were treated with 0.9% sodium citrate for 20 min, air dried and fixed with 70% ethanol on microscope slides. Labeled nuclei were examined using a Leitz DM RB microscope with epifluorescence illumination.

Statistical analysis of the data was carried out by analysis of the variance (ANOVA) with the aid of the SPSS/PC+ statistical package (Microsoft Co.). Duncan's test for multiple comparison between paired means (Sokal and Rohlf, 1979) was further applied to detect significant ($p \leq 0.05$) differences between groups.

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