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Parthenogenetic Activation of Porcine Oocytes by Calcium Ionophore A23187

Abstract
This study was designated to clarify the influence of activation of porcine matured oocytes by calcium ionophore on *in vitro* development of the parthenotes. The follicular oocytes were matured, activated and cultured in North Carolina State University-23 (NCSU-23) medium supplemented with 10% porcine follicular fluid (pFF). The *in vitro*-matured oocytes were exposed to calcium ionophore at concentrations of 12.5, 25 or 50 μM for 3, 5, 7 or 9 min. The activation rate of the oocytes increased as concentration of ionophore decreased, being at 27-33 and 68-77 % for the oocytes treated with 50 and 12.5 μM ionophore, respectively. Almost all activated oocytes were haploid. The highest cleavage rate (76%) and developmental rate to morula (41%) were observed in the oocytes treated with 12.5 μM ionophore for 5 min. However, development to blastocyst was observed only in the oocytes treated with 25 μM ionophore for 3 and 5 min (3 and 4% of treated oocytes, respectively). We concluded that the activation treatment of the porcine oocytes with 12.5 μM ionophore for 5 min provided the highest developmental rate to morula, but this treatment is not sufficient to overcome a developmental block at the morula stage.

Key Words: pig, oocytes, activation, calcium ionophore

Zusammenfassung
Titel der Arbeit: **Parthenogenetische Aktivierung von Schweine-Oozyten mit Calcium Ionophor A23187**
Diese Arbeit war darauf ausgerichtet, den Einfluss der Aktivierung von reifen Schweine-Oozyten durch Calcium Ionophor auf die *in vitro* Entwicklung von Parthenoten zu klären. Die follikulären Oozyten wurden in NCSU-23 (North Carolina State University-23) Nährboden gereift, aktiviert und gezüchtet, der mit 10% Follikelflüssigkeit vom Schwein (pFF) angereichert war. Die *in vitro* gereiften Oozyten wurden Calcium Ionophor ausgesetzt in Konzentrationen von 12,5, 25 und 50 μM für jeweils eine Zeitdauer von 3, 5, 7 und 9 Minuten. Mit abnehmender Konzentration von Ionophor nahm die Rate der Aktivierung von Oozyten zu. Sie betrug 27-33% beziehungsweise 68-77 % für die Konzentrationen von 50 beziehungsweise 12,5 μM Ionophor. Fast alle aktivierten Oozyten waren haploid. Die höchste Zellteilungsrate (76%) sowie die schnellste Entwicklung zur Morula (41%) wurde bei den Oozyten beobachtet, die mit Ionophor in der Konzentration von 12,5 μM für 5 Minuten behandelt wurden. Die Entwicklung zu Blastozyten wurde aber nur für die Oozyten beobachtet, die mit Ionophor in der Konzentration von 25 μM für 3 und 5 Minuten behandelt wurden (3% beziehungsweise 4% der behandelten Oozyten). Es wird geschlussfolgert, dass die Aktivierung von Schweine-Oozyten mit Ionophor in der Konzentration von 12,5 μM für 5 Minuten zwar die höchste Entwicklungsgeschwindigkeit zur Morula ermöglichte, diese Behandlung jedoch nicht ausreicht die Entwicklungsblockade im Stadium der Morula zu überwinden.

Schlüsselwörter: Schwein, Oozyten, Aktivierung, Calcium Ionophor

Introduction
Activation of mammalian oocytes by sperm penetration or parthenogenetic stimulation resumes meiotic division from the metaphase II stage (KAUFMAN, 1983). Artificial activation of the oocytes is important in the cloning of domestic animals using nuclear transfer, and the activation treatment is known to induce an increase of intracellular levels of free calcium ions (PRATHER, 2001; SUN and NAGAI, 2003). The elevated cytoplasmic levels of calcium ions in activated oocytes influence the activity of molecules of the maturation promoting factor (HASHIMOTO and KISHIMOTO,

1988) and the cytostatic factor (MASUI, 1991), which are responsible for the meiotic block at the metaphase II stage. Calcium ionophore A23187, ethanol and electrical pulse have been used as activation stimuli to increase calcium concentration in the ooplasm. Activation of *in vitro*-matured porcine oocytes has been induced by ionophore (HAGEN et al., 1991; WANG et al., 1998a, 1998b, 1999; JÍLEK et al., 2000, 2001; SUZUKI et al., 2002; SEDMÍKOVÁ et al., 2003) and the combination of ionophore and protein synthesis inhibitors (CHA et al., 1997; JÍLEK et al., 2000) or protein kinase inhibitors (JÍLEK et al., 2001). The ionophore treatment of the porcine oocytes is a simple method for induction of activation without expensive equipment, but results in a low *in vitro* development of the activated oocytes or parthenogenetic embryos (WANG et al., 1998a; JÍLEK et al., 2000, 2001; SEDMÍKOVÁ et al., 2003). Condition for the activation of porcine oocytes by ionophore was evaluated using modified Tris-buffered medium (WANG et al., 1998b, 1999) and modified Medium 199 (JÍLEK et al., 2000, 2001; SEDMÍKOVÁ et al., 2003). Porcine embryos (MIYANO et al., 1994) and parthenotes (KURE-BAYASHI et al., 1996), however, showed a low developmental potency in culture with the modified Medium 199. Therefore, the condition of ionophore treatment should be reevaluated using the medium optimal for *in vitro* culture of the porcine embryos.

In order to clarify the influence of the activation of porcine matured oocytes by calcium ionophore on *in vitro* development of the parthenotes, the concentration of ionophore and duration of the treatment were examined in the present study using NCSU-23 medium (PETTERS and WELLS, 1993) that was developed for the culture of the porcine embryos.

Materials and methods

Collection and *in vitro* maturation of porcine oocytes

Prepubertal pig oocytes (mainly Landrace × Large White × Duroc, approximately 6 months of age) were collected from a local slaughterhouse and transported to the laboratory in a physiological saline at 37°C within 4 hr of slaughter. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles 2-6 mm in diameter through an 18G needle attached to a 10-ml syringe and put into a HEPES-buffered Tyrode's medium (BAVISTER et al., 1983). The maturation medium was bovine serum albumin (BSA)-free NCSU-23 supplemented with 10% pFF, 0.57 mM cysteine (Sigma Chemical Co., St. Louis, MO, USA), 10 IU/ml equine chorionic gonadotropin (Serotropin, Teikoku Hormone Mfg. Co. Ltd., Tokyo, Japan) and 10 IU/ml human chorionic gonadotropin (Gonatropin, Teikoku Hormone). The pFF was collected from prepubertal pig ovarian follicles 4-6 mm in diameter, filtrated through 0.45-µm filters, and stored at -30°C until use. Ten to twelve COCs with evenly granulated ooplasm (>110 µm in diameter) and intact cumulus cell layers were put into a 50-µl drop of maturation medium, which was prepared under paraffin oil in a 60-mm plastic dish (Falcon 1007, Becton Dickinson Laboware, Lincoln Park, NJ, USA) and cultured for 20 hr at 38.5°C under humidified atmosphere of 5% CO_2 in air. The COCs were then transferred to a 50-µl drop of maturation medium without hormones and cultured for an additional 22 hr.

Activation of oocytes

Calcium ionophore A23187 (Sigma) was dissolved in dimethylsulfoxide (DMSO, Kanto Kagaku Co., Tokyo, Japan) at a concentration of 1.9 mM as a stock solution.

The stock solution was added to NCSU-23 medium with 10% pFF to make an activation medium with final concentrations of 12.5, 25 and 50 μM ionophore. After maturation culture of the oocytes, the cumulus cells were mechanically removed from the oocytes using a finely pulled Pasteur pipette. The oocytes with the first polar body in the perivitelline space were considered to be matured. The matured oocytes were cultured in the activation medium for 3-9 min.

Evaluation of oocyte activation

Oocytes were mounted on a glass slide, fixed with acetic acid and ethanol mixture (1:3, v/v) for at least 24 hr, and stained with 1% aceto-orcein. The slides were examined under a phase contrast microscope. The oocytes with a pronucleus and two polar bodies (haploids), those with two pronuclei and one polar body (diploids), and 2-cell eggs with immediate cleavage (haploid) were considered as activated, whereas the oocytes at the metaphase II to telophase II stages were classified into non-activated oocytes.

Experimental design

Experiment 1

The effect of the ionophore treatment on oocyte activation was examined. The matured oocytes were exposed to ionophore at concentrations of 12.5, 25 or 50 μM for 3, 5, 7 or 9 min. A total of 10-20 oocytes were put in a 50-μl drop of the activation medium, which was prepared under paraffin oil in a plastic dish. After the ionophore treatment, the oocytes were washed three times in a NCSU-23 medium without ionophore, and the oocytes were cultured in a 50-μl drop of NCSU-23 medium with 10% pFF for 24 hr at 38.5°C under humidified atmosphere of 5% CO_2 in air. The activation rate of the oocytes was then examined. In the control group, the oocytes were exposed to an ionophore-free medium containing the same amount of DMSO (0.07-0.26%) for the same amount of time.

Experiment 2

The effect of the ionophore treatment on *in vitro* development of activated oocytes or parthenotes was examined. The matured oocytes were exposed to ionophore at concentrations of 12.5, 25 or 50 μM for 3, 5, 7 or 9 min as in Experiment 1. After the ionophore treatment, the oocytes were washed in a NCSU-23 medium without ionophore, and 10-12 oocytes were cultured in a 50-μl drop of NCSU-23 medium with pFF for 144 hr at 38.5°C under humidified atmosphere of 5% CO_2 in air. Culture medium was exchanged every 24 hr. Developmental stage of the parthenotes and the number of cells in cleaving parthenotes were examined at the end of culture. The number of nuclei in parthenotes was counted by the same method used for evaluation of oocyte activation (Experiment 1).

Statistical analysis

Each experiment was carried out 4-7 times, and the results were pooled for presentation and statistical analysis. The activation and developmental rates of the oocytes among the ionophore-treatment groups were compared by Fisher's exact test with Stat View 4.0 (Abacus Concepts, Berkeley, CA, USA). A probability level of $P<0.05$ was considered statistically significant.

Results

Experiment 1

In vitro maturation rate of porcine oocytes was 68%. Activation rates of the porcine matured oocytes that were treated with ionophore are shown in Table 1. No activation was occurred in the control group (0/88 oocytes). The activation rate increased as concentration of ionophore decreased, being at 27-33 and 68-77 % for the oocytes treated with 50 and 12.5 µM ionophore, respectively. In the 12.5 and 25 µM-ionophore treatments, duration of the treatment did not affect the oocyte activation. In 50 µM-ionophore treatments, however, the oocytes treated for 5-9 min showed significantly lower activation rates than those treated for 3 min ($P<0.05$). Almost all activated oocytes were haploid.

Table 1
Activation rates of porcine matured oocytes treated with ionophore (Aktivierungsrate von reifen Schweine-Oozyten nach Behandlung mit Ionophor)

Concentration of ionophore (µM)	Duration of treatment (min)	No. of oocytes treated (n)	No. of activated oocytes (n) (%)	Classification of activated oocyte (n) (%) *		
				1N2PB (Haploid)	2N1PB (Diploid)	IC (Haploid)
12.5	3	90	68 (76) [a]	67 (98)	0 (0)	1 (2)
	5	85	65 (77) [a]	64 (98)	0 (0)	1 (2)
	7	97	69 (71) [a]	67 (96)	1 (2)	1 (2)
	9	87	59 (68) [ab]	54 (92)	1 (2)	4 (6)
25.0	3	49	30 (61) [ab]	28 (94)	1 (3)	1 (3)
	5	64	34 (53) [bc]	32 (94)	1 (3)	1 (3)
	7	49	26 (53) [bc]	24 (92)	0 (0)	2 (8)
	9	43	23 (54) [bc]	22 (96)	0 (0)	1 (4)
50.0	3	39	13 (33) [c]	13 (100)	0 (0)	0 (0)
	5	41	13 (32) [d]	13 (100)	0 (0)	0 (0)
	7	52	15 (29) [d]	15 (100)	0 (0)	0 (0)
	9	52	14 (27) [d]	14 (100)	0 (0)	0 (0)

Experiments were repeated 4 times. * 1N2PB: one pronucleus and two polar bodies; 2N1PB: two pronuclei and one polar body; IC: immediate cleavage. Data with different superscripts differ significantly ($P<0.05$).

Experiment 2

Development of the porcine oocytes that were activated by ionophore is shown in Table 2. The oocytes in the control group did not develop to the 2-cell stage (0/99 oocytes) and degenerated. The percentage of oocytes that developed to or beyond the 2-cell stage (cleavage rate) increased as concentration of ionophore decreased and duration of the treatment shortened. The highest cleavage rate (76%) was observed in the oocytes treated with 12.5 µM ionophore for 5 min. Furthermore, the developmental rate to morula in the oocytes treated with 12.5 µM ionophore for 5 min (41%) was significantly higher than those in the other treatments ($P<0.05$). However, the development of the parthenotes was blocked at the morula stage. Number of nuclei in the morula was 16-32. Development to blastocyst was observed only in the oocytes

treated with 25 µM ionophore for 3 and 5 min (3 and 4% of treated oocytes, respectively). Number of nuclei in the blastocyst was 37.7±1.8 (mean ± SD, n=7).

Table 2
In vitro development of porcine oocytes after treatment with ionophore (In vitro Entwicklung von Schweine-Oozyten nach Behandlung mit Ionophor)

Ionophore treatment		No. of oocytes treated (n)	No. of oocytes developed to ≥2-cell stage (n) (%)	% of oocytes developed to each stage (%)*				
Concentration (µM)	Duration (min)			2-cell	4-cell	8-cell	Morula	Blast
12.5	3	65	47 (72) ^{ab}	11 ^{ab}	23 ^{a}	15 ^{a}	23 ^{b}	0 ^{ab}
	5	63	48 (76) ^{a}	5 ^{abcd}	18 ^{ab}	11 ^{a}	41 ^{a}	0 ^{ab}
	7	64	37 (58) ^{b}	9 ^{ab}	9 ^{ace}	25 ^{b}	14 ^{bcd}	0 ^{ab}
	9	66	39 (59) ^{b}	14 ^{a}	18 ^{ab}	11 ^{a}	17 ^{bc}	0 ^{ab}
25.0	3	152	62 (41) ^{c}	5 ^{abcd}	8 ^{ce}	11 ^{a}	14 ^{bcd}	3 ^{ac}
	5	165	54 (33) ^{cd}	6 ^{abc}	8 ^{c}	9 ^{a}	5 ^{ef}	4 ^{a}
	7	164	55 (34) ^{c}	5 ^{abc}	10 ^{bc}	8 ^{a}	10 ^{cde}	0 ^{b}
	9	130	42 (32) ^{cd}	5 ^{bcd}	8 ^{cd}	11 ^{a}	9 ^{cde}	0 ^{bc}
50.0	3	154	36 (23) ^{de}	2 ^{cd}	6 ^{cd}	8 ^{a}	7 ^{de}	0 ^{b}
	5	164	32 (20) ^{ef}	1 ^{d}	5 ^{cd}	8 ^{a}	5 ^{ef}	0 ^{b}
	7	165	24 (15) ^{f}	2 ^{cd}	2 ^{d}	8 ^{a}	2 ^{fg}	0 ^{b}
	9	121	13 (11) ^{f}	1 ^{cd}	2 ^{de}	6 ^{a}	0 ^{g}	0 ^{bc}

Experiments were repeated 4-7 times. * No. of oocytes developed / No. of oocytes treated. Data with different superscripts differ significantly ($P<0.05$).

Discussion

The present study confirmed that porcine parthenotes derived from activation by calcium ionophore could develop to the blastocyst stage by using NCSU-23 medium with pFF for *in vitro* maturation, activation and culture. However, a developmental block of the parthenotes had occurred at the morula stage and resulted in a low developmental rate to the blastocysts with small number of cells.

In the present study, a high activation rate (68-77%) was obtained by 12.5 µM ionophore treatment. This value is similar to that previously reported in the porcine oocytes activated by calcium ionophore. The highest activation rate was reported in the porcine oocytes treated with 25-100 µM ionophore for 5 min (HAGEN et al., 1991) or 50 µM ionophore for 2-7 min (WANG et al., 1999; JÍLEK et al., 2000, 2001). The activation rate of the porcine oocytes increased as concentration of ionophore increased from 10 to 100 µM (WANG et al., 1998b). In the present study, however, the activation rate decreased as concentration of ionophore increased from 12.5 to 50 µM. The reason for this discrepancy is not clear. In the ionophore treatment of porcine oocytes, serum-free media (FUNAHASHI et al., 1994; JÍLEK et al., 2000, 2001; SUZUKI et al., 2002; SEDMÍKOVÁ et al., 2003) and media supplemented with BSA (WANG et al., 1998a, 1998b, 1999) were used as the activation media. Supplementation with polyvinyl alcohol instead of BSA in the activation medium inhibited pronuclear formation in the porcine oocytes (RUDDOCK et al., 2001). The present activation medium contained pFF, so that some proteins or molecules in the pFF might enhance the effect of ionophore.

In vitro culture of the porcine parthenotes derived from treatment with 12.5 μM ionophore for 5 min showed the highest cleavage rate (76%) and developmental rate to morula (41%). The developmental rate to morula in the porcine oocytes activated by calcium ionophore was improved in the present study because it was reported to be 1-8% (JÍLEK et al., 2000, 2001; SEDMÍKOVÁ et al., 2003). However, development to blastocyst was not observed in the treatment with 12.5 μM ionophore for 5 min but in the treatment with 25 μM ionophore for 3-5 min. This result indicates that *in vitro* development of the porcine parthenotes might be affected not only by the ionophore treatment but also the culture condition.

For *in vitro* culture of porcine embryos, various media such as the modified Whitten medium (BECKMANN and DAY, 1993), NCSU-23 medium (PETTERS and WELLS, 1993), modified Chatot, Ziomek, Bavister (CZB) medium (POLLARD et al., 1995) and Beltsville embryo culture medium (BECM)-3 (DOBRINSKY et al., 1996) have been used. KURE-BAYASHI et al. (1996) reported that the Whitten medium was superior to the modified Medium 199 for *in vitro* culture of the porcine parthenotes. When the porcine parthenotes derived from the ionophore treatment were cultured in modified Medium 199, their development was blocked at an early stage (2-4 cell stage, JÍLEK et al., 2000, 2001; SEDMÍKOVÁ et al., 2003). In the present study using an NCSU-23 medium, a developmental block at the morula stage was observed. Although the culture media were not compared in this study, the stage that the developmental block occurred seemed to be affected by the media. The developmental rate to blastocyst in the porcine oocytes activated by ionophore was reported to be 1-5% (WANG et al., 1998a; JÍLEK et al., 2000, 2001; SEDMÍKOVÁ et al., 2003), so that it was not improved in the present study (3-4%). Recently, porcine zygote medium (PZM) was developed for *in vitro* culture of the porcine embryos (YOSHIOKA et al., 2002). Development of the porcine parthenotes might be improved by using the medium optimized for embryo culture.

Porcine oocytes activated by parthenogenetic stimulation were classified into haploid, diploid, oocytes with three or more pronuclei and others (HATA et al., 1996; KURE-BAYASHI et al., 1996). Treatment of the porcine oocytes with calcium ionophore resulted in 59-91% haploid oocytes with a pronucleus and two polar bodies (FUNAHASHI et al., 1994; WANG et al., 1998a, 1998b; SUZUKI et al., 2002). However, SEDMÍKOVÁ et al. (2003) reported the presence of 76% diploid oocytes with two pronuclei and a polar body after the ionophore treatment. In the present study, most parthenotes derived from the ionophore treatment were haploid. The number of pronuclei in the activated oocytes was influenced by the maturation media (FUNAHASHI et al., 1994) and the intensity and duration of electric pulse at stimulation (PROCHÁZKA et al., 1992). Therefore, pronuclear formation and ploidy of the porcine activated oocytes seemed to be affected by the condition of oocyte maturation and activation. Porcine haploid parthenotes had a lower developmental potency to morula and blastocyst compared to diploid parthenotes (KURE-BAYASHI et al., 1996). In the present study, low developmental rates and the developmental block at the morula stage might be caused by haploidy of the parthenotes, genetic transition from the maternal to the embryonic genome and/or genomic imprinting. *In vitro* development of the diploid parthenotes derived from the cytochalasin treatment following activation should be further examined.

Activation rate, cleavage rate and developmental rate to blastocyst of the porcine parthenotes activated by calcium ionophore were lower than those activated by electrical stimulation (WANG et al., 1998a; SUZUKI et al., 2002). Over 40% of porcine diploid parthenotes derived from cytochalasin treatment following the electrical stimulation developed to blastocyst (ZHU et al., 2002). These results indicate that ionophore cannot induce all processes necessary for full-valued activation of the porcine oocytes. In electrical activation, inhibitors of protein synthesis or protein kinase were used after the stimulation to suppress synthesis of the proteins responsible for maintenance of the oocytes in meiotic arrest at the metaphase II stage (BING et al., 2003). Use of cycloheximide and 6-dimethyl aminopurine with ionophore increased the activation rate of the porcine oocytes but did not improve development to blastocyst (JÍLEK et al., 2000, 2001). Combination of other protein or protein kinase inhibitors with ionophore should be evaluated.

In conclusion, the present study showed that the activation treatment of the porcine oocytes with 12.5 μM calcium ionophore for 5 min provided the highest developmental rate to morula. To overcome the developmental block at the morula stage and enhance development to blastocyst, culture condition of immature oocytes and parthenotes, ploidy of the activated oocytes, and combined treatment of ionophore with protein inhibitors are necessary to be examined.

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