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Activation of pig oocytes by intracytoplasmic injection of strontium and barium

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Summary

Ovulated mouse oocytes are activated by exposure to culture medium containing Sr^{2+} or Ba^{2+} or by intracytoplasmic injection of the divalent cations. It is known that *in vitro* matured pig oocytes are activated by the intracytoplasmic injection of Ca^{2+}. In this study, we examined the effect of exposure and of intracytoplasmic injection of Sr^{2+} or Ba^{2+} on *in vitro* matured pig oocytes (MII-oocytes). When MII-oocytes were exposed to the medium containing divalent cations, no oocytes were activated. However, in the case of oocytes that were injected with Sr^{2+}, Ba^{2+} and Ca^{2+}, at 6 h after injection, 64%, 71% and 86% of the oocytes had been released from MII-arrest, and 51%, 67% and 84% formed female pronuclei, respectively. The initial transient in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by the Ca^{2+} indicator dye fluo-4 dextran. Microinjection of Sr^{2+}, Ba^{2+} or Ca^{2+} induced a rapid elevation of $[Ca^{2+}]_i$. The exocytosis of cortical granules was examined by staining with fluorescein isothiocyanate (FITC)-labelled peanut agglutinin. After an injection of divalent cations, a release of cortical granules was observed in the oocytes. Maturation promoting factor (MPF) activity declined to a low level after 6 h in all the oocytes injected with divalent cations. To test their developmental ability, injected oocytes were treated with cytochalasin B and then cultured for 168 h in NCSU23 medium. After 168 h, 29% (Sr^{2+}), 29% (Ba^{2+}) and 51% (Ca^{2+}) of the oocytes had developed to the blastocyst stage. These results indicate that intracytoplasmic injection of Sr^{2+} and Ba^{2+}, like that of Ca^{2+}, induces *in vitro* matured pig oocytes to be released from MII-arrest and leads them into a series of events related to oocyte activation.

Key words: Activation, Barium, Calcium, Pig oocyte, Strontium

Introduction

Ovulated oocytes remain arrested at metaphase II until their activation is induced by fertilising spermatozoa or artificial stimuli. Activation events include the exocytosis of cortical granules, resumption and completion of meiosis II with the extrusion of the second polar body, and pronuclear formation. The meiotic resumption of the oocyte is induced by the inactivation of a maturation promoting factor (MPF; Masui & Markert, 1971), which had been activated during oocyte maturation.

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All the activation events of the oocyte have been thought to be triggered by a transient in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and subsequent Ca^{2+} oscillations (Cuthbertson & Cobbold, 1985; Collas *et al*., 1993; Swann & Ozil, 1994). Evidence for a critical role of Ca^{2+} in oocyte activation comes from studies showing oocyte activation by artificial incrementation of $[Ca^{2+}]_i$. Microinjection of Ca^{2+} into oocytes and application of the Ca^{2+} ionophore A23187 both trigger parthenogenetic activation and pronuclear formation of oocytes, including pig oocytes (Steinhardt *et al*., 1974; Fulton & Whittingham, 1978; Kline & Kline, 1992). The finding that other parthenogenetic agents cause a $[Ca^{2+}]_i$ increase in mammalian oocytes suggests that Ca^{2+} is both a necessary and a sufficient trigger for oocyte activation (Swann & Ozil, 1994).

Artificial activation of oocytes has been applied in the field of oocyte- or embryo-related technologies, including intracytoplasmic sperm injection (ICSI) for

the treatment of male infertility conditions such as azoospermia (Van Steirteghem *et al*., 1993) as well as animal cloning by nuclear transfer (Wilmut *et al*., 1997; Wakayama *et al*., 1998). Oocyte activation studies have also highlighted a number of problems in understanding the relationship between $[Ca^{2+}]_i$ dynamics and events concerning oocyte activation. After fertilisation, mammalian oocytes show Ca^{2+} oscillations. In contrast, most of the parthenogenetic agents cause a monotonic increase in $[Ca^{2+}]_i$ (Cuthbertson *et al*., 1981; Swann & Ozil, 1994). A clear exception to this rule is Sr^{2+} in the medium, which induces Ca^{2+} oscillations in mouse oocytes (Kline & Kline, 1992; Bos-Mikich *et al*., 1995). In the first experiment of the present study we used medium containing Sr^{2+} or Ba^{2+} to induce the activation of *in vitro* matured pig oocytes. In the second experiment, pig oocytes were activated by intracytoplasmic injection of Sr^{2+} or Ba^{2+}, and we examined a series of events related to oocyte activation: the increase in $[Ca^{2+}]_i$, cortical granule exocytosis, meiotic resumption, MPF inactivation, pronuclear formation, and development to the blastocyst stage.

Materials and methods

Oocyte maturation

Pig ovaries were obtained from prepubertal gilts at a local slaughterhouse. Following three washes in Dulbecco's phosphate-buffered saline containing 0.1% polyvinyl alcohol (PBS-PVA), intact healthy antral follicles 4.0–6.0 mm in diameter were dissected in PBS-PVA from ovaries using the technique described by Moor & Trounson (1977). Follicles were opened in HEPES-buffered medium 199 (Nissui Pharmaceutical, Tokyo, Japan), and oocyte–cumulus–granulosa cell complexes (OCGCs) were isolated from the follicles. After two washes, OCGCs were cultured for 45–48 h in 2 ml of bicarbonate-buffered medium 199 supplemented with 10% fetal bovine serum (FBS; Dainippon Pharmaceutical, Osaka, Japan), 0.1 mg/ml sodium pyruvate, 0.08 mg/ml kanamycin sulfate, 0.1 IU/ml human menopausal gonadotropin (hMG; Pergonal, Teikokuzoki, Tokyo, Japan), and two everted theca shells with gentle agitation. The culture of OCGCs was carried out in an atmosphere of 5% CO_2 in humidified air at 38.5 °C. After culture, OCGCs were recovered and treated with 0.01% hyaluronidase (Sigma Chemical, St Louis, MO) to remove cumulus cells. Oocytes were then denuded completely by pipetting with a small-bore pipette. Oocytes extruding the first polar body were selected to be used as matured oocytes (MII-oocytes) in the following experiments.

Oocyte activation

In all the experiments with the exception of the developmental experiment, two modified TALP media (Bavister *et al*., 1983) were used for the micromanipulation and culture of oocytes. Modified bicarbonate-TALP medium (TL-PVA) and HEPES-buffered TALP medium (HEPES-TL-PVA) did not contain bovine serum albumin (BSA) and sodium pyruvate and were supplemented with 0.1% PVA.

In the first experiment, MII-oocytes were exposed to medium containing three different divalent cations: Sr^{2+}, Ba^{2+} and Ca^{2+}. In this experiment, $CaCl_2$ and $MgCl_2$ were omitted from HEPES-TL-PVA, and $NaH_2PO_4{\cdot}H_2O$ and bicarbonate were also omitted in order to prevent the ionic precipitation (modified HEPES-TL-PVA). MII-oocytes were incubated in modified HEPES-TL-PVA containing 0, 1, 5, or 10 mM $SrCl_2$, $BaCl_2$ or $CaCl_2$ at 38.5 °C for 2 h. Then the oocytes were washed three times in TL-PVA and further cultured in TL-PVA for 4 h. As a control, oocytes were cultured in TL-PVA for 2 h and further in new TL-PVA for 4 h.

In the second experiment, MII-oocytes were microinjected with divalent cations. Oocytes were injected with 8.2–14.1 pl (0.9–1.6% of the oocyte volume) of 0.1 M $SrCl_2$, $BaCl_2$ or $CaCl_2$ in 10 mM HEPES buffered solution (pH 7.4) to make a final concentration of 0.5–1 mM in the injected oocytes. They were further cultured in TL-PVA for 6 h. Microinjections were performed in Ca^{2+}- and Mg^{2+}-free HEPES-TL-PVA. As a control, HEPES-buffered solution was injected into MII-oocytes.

In both experiments, the oocytes were mounted on slides after the culture, fixed in an acetic acid–ethanol (1:3, v/v) solution, stained with 1% aceto-orcein, and observed under a Nomarski interference microscope. Activation was assessed by the meiotic resumption of the oocytes from metaphase II (MII), and their pronuclear formation was also examined.

$[Ca^{2+}]_i$ measurement

MII-oocytes were microinjected with the Ca^{2+} indicator dye, 1 mM fluo-4 dextran (Molecular Probes, Eugene, OR), in 10 mM HEPES-buffered solution to make a final concentration of 5–10 μM in the injected oocytes. At 30 min after fluo-4 injection, the oocytes were injected with $SrCl_2$ (n = 8), $BaCl_2$ (n = 8) or $CaCl_2$ (n = 10) in 10 mM HEPES-buffered solution in the same manner as described above under a confocal laser scanning microscope, and the change in $[Ca^{2+}]_i$ was measured at 38.5 °C for 10 min. The confocal microscope was equipped with a laser unit LSM-LU-100, excitation filter DM 488 and emission filter BP535 (Olympus Optical, Tokyo, Japan). As a control, HEPES-buffered solution was injected into fluo-4-injected MII-oocytes in the same manner.

Staining of cortical granules

Cortical granules of oocytes were labelled as described previously (Katayama *et al*., 2002). Briefly, MII-oocytes were injected with $SrCl_2$ (n = 10), $BaCl_2$ (n = 11) or $CaCl_2$ (n = 12), and cultured for 6 h in HEPES-TL-PVA as described above. After the culture, the oocytes were fixed in 3% paraformaldehyde in PBS-PVA for 30 min at room temperature. After being rinsed twice in 10% BSA in PBS (PBS-BSA), the oocytes were kept in PBS-BSA at least overnight. They were then treated with 0.1% Triton X-100 (Sigma) in PBS for 5 min and rinsed twice in PBS-BSA. The oocytes were incubated in 20 μg/ml fluorescein isothiocyanate (FITC)-labelled peanut agglutinin (FITC-PNA; Sigma) in PBS-PVA for 30 min. Following rinsing in PBS-BSA, DNA was counterstained with 400 μg/ml propidium iodide (PI; Sigma) for 15–20 min. After rinsing, oocytes were mounted in an anti-fade medium (Vector Laboratories, Burlingame, CA) and observed under a confocal laser-scanning microscope (MRC 1024 system; Bio-Rad, Hercules, CA). As the control, non-treated MII-oocytes and oocytes injected with HEPES-buffered solution were treated and observed in the same manner.

Histone H1 kinase assay

MPF activity was measured using histone H1 as a substrate. Groups of two oocytes were recovered 6 h after the injection of divalent cations. After three washes in PBS-PVA, the oocytes were transferred into Eppendorf tubes with 1 μl of PBS-PVA and 4 μl of ice-cold extraction buffer, and stored at –80 °C before the kinase assay. The kinase buffer was composed of 80 mM β-glycerophosphate, 25 mM HEPES (pH 7.2), 10 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM PMSF, 0.1 mM Na_3VO_4, 1 μg/ml aprotinin (Sigma) and 1 μg/ml leupeptin (Sigma) (Nebreda *et al*., 1995).

After thawing, the oocytes were centrifuged at 13 000 *g* for 2 min. To measure MPF activity, 5 μl of kinase buffer and 5 μl of histone H1 solution (4.25 mg/ml from calf thymus; Boehringer, Tokyo, Japan) were added and incubated for 20 min at 37 °C. The kinase buffer was composed of 75 mM β-glycerophosphate, 75 mM HEPES (pH 7.2), 0.1 mM EGTA, 75 mM $MgCl_2$, 6 mM DTT, 10 mM ATP, 15 μM cAMP-dependent protein kinase inhibitor peptide (sequence: TTYADFIASGRTGRRNAIHD, Sigma) and 0.3 μCi/μl [γ-^{32}P]ATP (250 μCi/25 μl, Amersham Pharmacia Biotech, UK). The reaction was terminated by the addition of 5 μl of 4× concentrated SDS sample buffer (Laemmli, 1970). The samples were boiled for 5 min and then loaded onto 13% SDS-polyacrylamide gels to separate the labelled histone H1. After being run, the gels were dried and autoradiographed. The experiment was repeated three times. As the control, oocytes at the germinal vesicle stage, MII-oocytes and oocytes injected with HEPES buffered solution were used.

Development of activated oocytes

The developmental ability of the pig oocytes was examined after the injection of divalent cations. The injected oocytes were cultured for 4 h in NCSU23 medium (Petters, 1992) containing 5 μg/ml cytochalasin B (Sigma) and 4 mg/ml BSA, and further cultured for 168 h in NCSU23 medium with 4 mg/ml BSA. After 48 h of culture, the rate of cleaved oocytes was examined. After 168 h the development of the oocytes to the blastocyst stage was evaluated. The blastocysts were stained with 2 μg/ml Hoechst 33342 (Polysciences, Warrington, PA), and the number of nuclei was determined by epifluorescence microscopy (Olympus).

Statistical analysis

The percentages of oocytes in the different treatment groups that resumed meiosis, formed pronuclei and developed to the blastocyst stage, as well as the nuclear number of blastocysts and the duration of the Ca^{2+} peak, were analysed using a one-way analysis of variance (ANOVA). The significance of differences was assessed using Dunn's procedure as a multiple comparison procedure. A p value < 0.05 was considered statistically significant.

Results

Injection of Sr^{2+}, Ba^{2+}, and Ca^{2+} induces activation of pig oocytes

After pig oocytes were exposed to the medium containing (24–35 oocytes in each group) 1, 5 or 10 mM Sr^{2+}, Ba^{2+} and Ca^{2+}, no oocyte was activated in any of the experimental groups. However, these concentrations of Sr^{2+} and Ba^{2+} in the medium induced the activation of mouse ovulated oocytes (data not shown).

In the next experiment, pig oocytes were directly injected with Sr^{2+}, Ba^{2+} or Ca^{2+}. At 6 h after injection with Sr^{2+}, Ba^{2+} and Ca^{2+}, 64%, 71% and 86% of the oocytes were released from MII-arrest, and 51%, 67% and 84% formed female pronuclei, respectively (Table 1). The control oocytes injected with HEPES buffer resumed meiosis at a significantly lower rate ($p < 0.05$).

Change in $[Ca^{2+}]_i$ by microinjection of divalent cations into pig oocytes

After microinjection of Sr^{2+}, Ba^{2+} or Ca^{2+}, an elevation of $[Ca^{2+}]_i$ was induced in all the examined oocytes (Sr^{2+}: 8/8, Ba^{2+}: 8/8, Ca^{2+}: 10/10). The typical patterns of the

Table 1 Activation of pig oocytes after microinjection of divalent cations

Divalent cations injected	No. of oocytes injected	No. (%) of oocytes resuming meiosis	No. (%) of oocytes having pronucleus
Sr^{2+}	47	30 (64)[a]	24 (51)[a]
Ba^{2+}	51	36 (71)[a]	34 (67)[a]
Ca^{2+}	49	42 (86)[b]	41 (84)[b]
Control*	47	2 (4)[c]	2 (4)[c]

*HEPES-buffered solution.
[a–c]Values within a column having different superscripts are significantly different ($p < 0.05$).

transient in $[Ca^{2+}]_i$ of the oocytes are shown in Fig. 1. An increase in $[Ca^{2+}]_i$ took place immediately upon injection. The mean duration ± SD of the transient in the oocytes injected with Sr^{2+}, Ba^{2+} and Ca^{2+} was 52.2 ± 21.3, 446.2 ± 69.5, and 65.6 ± 30.8 s, respectively. The duration of the transient in the oocytes injected with Ba^{2+} was significantly longer than in those oocytes injected with Sr^{2+} and Ca^{2+} ($p < 0.05$). After the transient, no subsequent increase in or oscillations of $[Ca^{2+}]_i$ were observed in any of the injected oocytes up to 30 min after injection (data not shown).

Exocytosis of cortical granules by injection of divalent cations

In MII-oocytes, cortical granules were localised just under the plasma membrane as a bright continuous ring. In the control oocytes injected with 10 mM HEPES buffer (6/6), the cortical granules showed a similar distribution after 6 h (Fig. 2*d*). On the other hand, in the oocytes injected with Sr^{2+} (10/10), Ba^{2+} (11/11) and Ca^{2+} (11/12), the ring of cortical granules became discontinuous, and patches of cortical granule material were observed on the oocyte surface (Fig. 2*a–c*). The distribution patterns were similar in all the oocytes injected with three different divalent cations.

MPF activity after injection of divalent cations

MPF activity was low in the oocytes at the germinal vesicle stage, and high in MII-oocytes. After 6 h, the activity declined in all the experimental groups injected with divalent cations, although the activity remained higher in the control oocytes than in the experimental groups (Fig. 3).

Development of oocytes injected with divalent cations

Of the oocytes injected with Sr^{2+}, Ba^{2+} and Ca^{2+}, 53%, 59% and 80% cleaved, respectively (Table 2). These

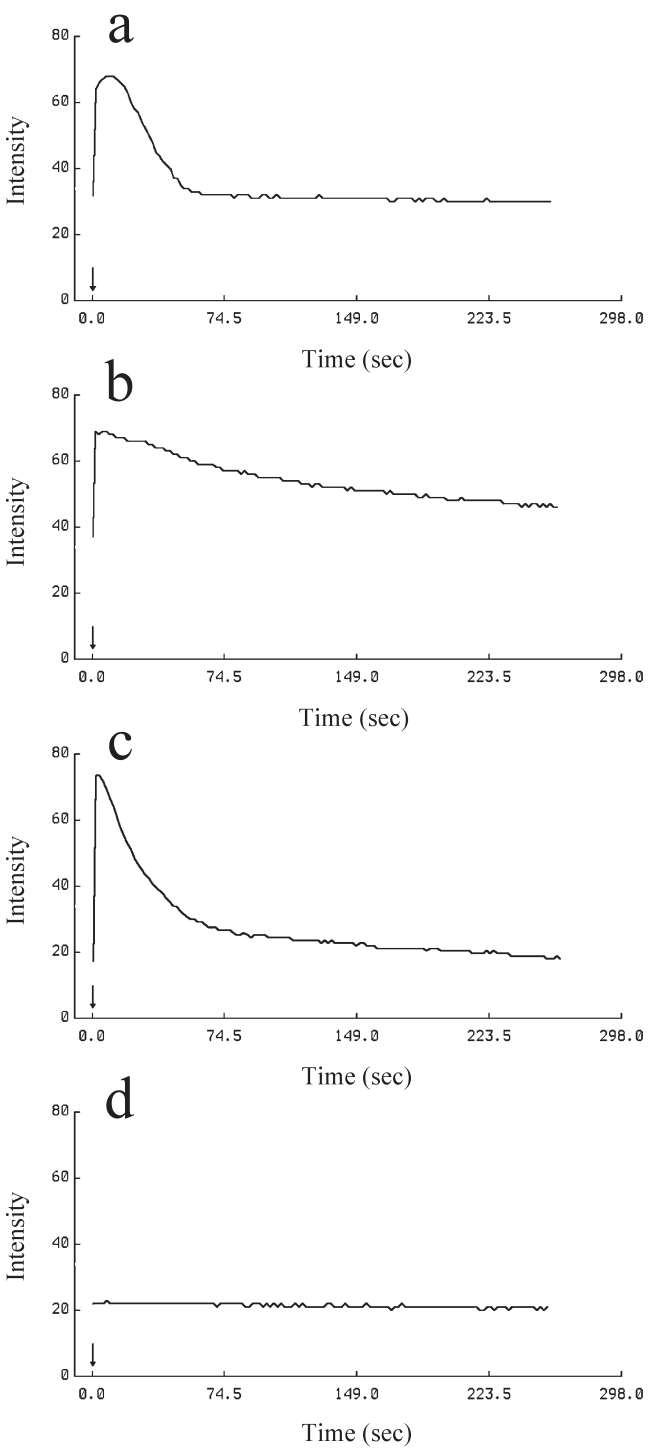

Figure 1 Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) following the injection of divalent cations. Pig oocytes cultured for 45–48 h were injected with 1 mM fluo-4 dextran, as a $[Ca^{2+}]_i$ indicator dye, and then 8.2–14.1 pl of 0.1 M Sr^{2+} (*a*), Ba^{2+} (*b*) or Ca^{2+} (*c*) was injected into the oocytes. Changes in $[Ca^{2+}]_i$ were measured under the confocal microscope. Control oocytes were injected with 10 mM HEPES-buffered solution (*d*). The arrow marks the time of injection.

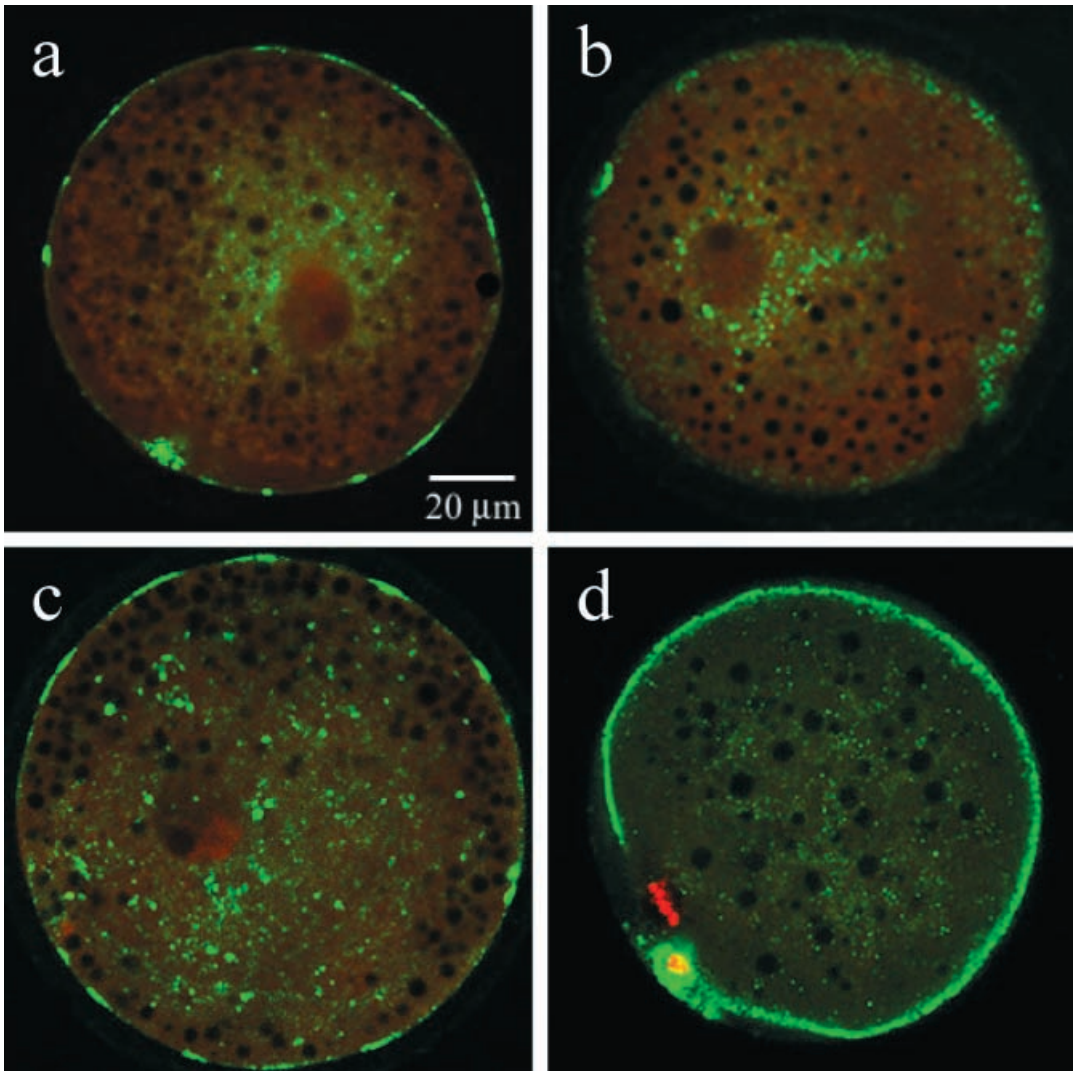

Figure 2 Confocal images of cortical granules of pig oocytes stained with FITC-labelled peanut agglutinin. Pig oocytes cultured for 45–48 h were injected with 8.2–14.1 pl of 0.1 M Sr^{2+} (*a*), Ba^{2+} (*b*) or Ca^{2+} (*c*) and were further cultured in TL-PVA medium for 6 h. After culture, the oocytes were fixed, stained and examined for cortical granule exocytosis. Control oocytes were injected with 10 mM HEPES-buffered solution and stained in the same manner (*d*).

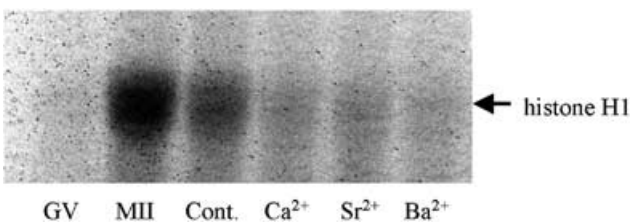

Figure 3 Changes in the activities in MPF (histone H1 kinase) at 6 h after the injection of divalent cations. Pig oocytes cultured for 45–48 h were injected with 8.2–14.1 pl of 0.1 M Sr^{2+}, Ba^{2+} or Ca^{2+} and further cultured in TL-PVA medium for 6 h. After the culture, the MPF activities of the oocytes were detected by phosphorylation of histone H1. GV, germinal vesicle; MII, metaphase II; Cont., HEPES-buffered solution.

Table 2 *In vitro* devleopment in pig oocytes after injection of divalent cations

Divalent cations injected	No. of oocytes injected	No. (%) of oocytes cleaved	No. (%) of oocytes forming blastocyst
Sr^{2+}	51	27 (53)[a]	15 (29)
Ba^{2+}	51	30 (59)[a]	15 (29)
Ca^{2+}	50	40 (80)[a]	26 (51)
Control*	42	4 (10)[b]	0

*HEPES-buffered solution.
[a–c]Values within a column having different superscripts are significantly different ($p < 0.05$).

percentages were significantly higher than that in the control group (10%, $p < 0.05$). After 168 h, 29%, 29% and 51% of the oocytes injected with Sr^{2+}, Ba^{2+} and Ca^{2+} developed to the blastocyst stage, respectively, while none of the 42 control oocytes developed to blastocysts. The mean cell numbers of the blastocysts that developed from the oocytes injected with Sr^{2+}, Ba^{2+} and Ca^{2+} were 35 ± 17, 27 ± 15 and 35 ± 15, respectively.

Discussion

The results of the present experiments show that pig oocytes are activated by the intracytoplasmic injection of Sr^{2+} or Ba^{2+}, and they not only complete meiosis but also undergo a series of activation events including a transient increase in $[Ca^{2+}]_i$, cortical granule exocytosis, formation of a female pronucleus and MPF inactivation in a manner analogous to that observed in the oocytes injected with Ca^{2+} which were reported on previously (Macháty *et al*., 1996). However, no pig oocytes were activated in the medium supplemented with Sr^{2+} or Ba^{2+} in our experiment. On the other hand, Sr^{2+} or Ba^{2+} in the culture medium activates ovulated mouse oocytes (Whittingham & Siracusa, 1978).

The differences between mouse and pig oocytes are due to the functional differences related to the influx and efflux of divalent cations, including Ca^{2+}. In the mouse, external divalent cations enter the oocytes and induce the release of Ca^{2+} from intracellular Ca^{2+} stores, and in turn the released Ca^{2+} induces the serial events of oocyte activation (Kline & Kline, 1992). In the pig, $[Ca^{2+}]_i$ may not reach a threshold level in the oocytes by exposure to medium containing the divalent cation. It is thought that the permeability of the plasma membrane for divalent cations of pig oocytes is lower than that of the mouse. Alternatively, the divalent cations can enter the oocytes, although they may be rapidly pumped from the cytoplasm by the active transport to internal Ca^{2+} stores and/or outside of the oocytes before reaching the threshold level of $[Ca^{2+}]_i$. The results in the present study clearly show that the cations reached the threshold level in pig oocytes injected with the divalent cations. Sr^{2+} and Ba^{2+} worked efficiently, like Ca^{2+}, as the injected oocytes were activated and a series of activation events was initiated. Confocal microscopy images supported the transient increase in $[Ca^{2+}]_i$ in the pig oocytes injected with Sr^{2+}, Ba^{2+} and Ca^{2+}. In numerous cell types including oocytes, the increase in $[Ca^{2+}]_i$ can be generated either by a Ca^{2+} release from internal stores or by the opening of Ca^{2+} channels in the plasma membrane (Tsien & Tsien, 1990). Moreover, Sr^{2+} and Ba^{2+} have been reported to be substitutable for Ca^{2+} in Ca^{2+}-sensitive Ca^{2+} channels on the surface of intracellular Ca^{2+} stores in the *Xenopus* oocyte (Yao & Parker, 1992).

In mouse oocytes, the Sr^{2+} treatment generates an increase in $[Ca^{2+}]_i$ and subsequent repetitive increases in $[Ca^{2+}]_i$ (Ca^{2+} oscillations) (Kline & Kline, 1992; Bos-Mikich *et al*., 1995). In mouse (Swann, 1994) and hamster (Igusa & Miyazaki, 1983), Ca^{2+}-injected oocytes cause a single increase in $[Ca^{2+}]_i$. Pig oocytes injected with Sr^{2+}, Ba^{2+} or Ca^{2+} immediately induced a transient increase in $[Ca^{2+}]_i$ after injection, and Ca^{2+} oscillations were never observed in any of the injected oocytes.

Pig oocytes show cortical granule exocytosis within 6 h after the insemination of spermatozoa *in vitro* (Yoshida *et al*., 1993). Typically, the release of the cortical granules is induced by the elevated $[Ca^{2+}]_i$ caused by the sperm, but in several species it can be triggered by a wide range of treatments, such as electro-stimulus in the presence of external Ca^{2+} (Sun *et al*., 1992) and microinjection of Ca^{2+} (Macháty *et al*., 1996) in the pig, calcium ionophores in the hamster (Steinhardt *et al*., 1974), and exposure to medium containing Sr^{2+} in the mouse (Kline & Kline, 1992). In the present study, cortical granules were released within 6 h after the injection of Sr^{2+} or Ba^{2+} in pig oocytes, a result that is consistent with that previously reported using Ca^{2+}. The pathway to cortical granule exocytosis by injection of Sr^{2+} and Ba^{2+} is thought to include increases in $[Ca^{2+}]_i$ as described above.

Ca^{2+} injection induced the inactivation of histone H1 kinase. Histone H1 kinase activity is an indicator of MPF activity. MPF is composed of $p34^{cdc2}$ and cyclin B and was first described as an activity present in the metaphase cytoplasm of frog oocytes (Masui & Markert, 1971). Fertilisation or parthenogenetic activation induces a transient increase in $[Ca^{2+}]_i$ that leads to cyclin B degradation to inactivate MPF. The inactivation allows oocytes to escape from MII arrest and initiate embryo development. In our experiment, MPF activity decreased at 6 h after the injection of Sr^{2+} and Ba^{2+}. Sr^{2+} and Ba^{2+} may indirectly induce MPF inactivation via an increase in the $[Ca^{2+}]_i$ of the oocytes.

It has been reported in the mouse that Ca^{2+}-injected oocytes developed to blastocysts *in vitro* (Fulton & Whittingham, 1978), and also that Sr^{2+}-treated oocytes effectively developed to the blastocyst stage *in vivo* (Bos-Mikich *et al*., 1997). Macháty *et al*. (1996) reported that Ca^{2+}-injected pig oocytes developed to the blastocyst stage *in vivo*. In the present study, a relatively high percentage of *in vitro* matured pig oocytes injected with Sr^{2+} or Ba^{2+} developed to blastocysts (29%). The reason for the effective development is thought to be that the oocytes were treated with cytochalasin B to prevent the extrusion of the second polar body, although the injection of such divalent cations induced second polar body extrusion resulting in haploid embryos without cytochalasin B. The diploid embryos then may have had a better chance to develop to the blastocyst stage (Kure-bayashi *et al*., 1996).

In conclusion, the injection of Sr^{2+} or Ba^{2+} activates MII-arrested pig oocytes via an increase in $[Ca^{2+}]_i$ probably from intracellular Ca^{2+} stores in a manner similar to that observed for Ca^{2+}, and the increase in $[Ca^{2+}]_i$ leads the oocytes to a series of events related to oocyte activation.

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