Zygote **11** (August), pp 253–260. © 2003 Cambridge University Press
DOI:10.1017/S0967199403002302 Printed in the United Kingdom

Ultrastructural localisation of calcium deposits in pig oocytes maturing *in vitro*: effects of verapamil

J. Rozinek[1], *Z. Vaňourková*[2], *M. Sedmíková*[1], *V. Lánská*[1], *J. Petr*[3], *R. Rajmon*[1] *and F. Jílek*[1]
Czech University of Agriculture in Prague, Institute for Clinical and Experimental Medicine, and Research Institute of Animal Production, Prague, Czech Republic

Date submitted: 14.4.03. Date accepted: 15.5.03

Summary

The culture of pig oocytes in the presence of the calcium channel blocker verapamil (0.02 mM) resulted in the blocking of meiosis at the metaphase I stage, and only a small fraction (about 28%) of the oocytes were able to continue their maturation to the stage of metaphase II. Hence, meiotic maturation in pig oocytes is a calcium-dependent process. After isolation of the pig oocytes from their follicles, the intracellular calcium deposits in the oocyte and granulosa cells, detectable using the combined oxalate–pyroantimonate method, are depleted. The amount of calcium deposits in the oocyte and granulosa cells increased during oocyte meiotic maturation *in vitro,* especially in the nucleus, mitochondria, vacuoles and cytoplasm. The replenishment of calcium deposits is significantly changed under the effect of verapamil. The increase in calcium deposits in the oocyte nucleus was delayed, a much larger amount of deposits was formed in the mitochondria, and the amount of deposits in the vacuoles was demonstrably smaller. A significant peak in the accumulation of calcium deposits was observed in the cytoplasm of verapamil-treated oocytes after 16 h of *in vitro* culture. We propose that an altered pattern in the replenishment of calcium deposits can disturb intracellular signalling and prevent the exit of oocytes from the metaphase I stage.

Keywords: Calcium deposits, Maturation in vitro, Oocyte, Pig, Verapamil

Introduction

Calcium ions are common messengers in intracellular signalling (for review see Clapham, 1995), and these ions play an active role in the regulation of meiotic maturation in mammalian oocytes. From the point of view of developmental biology, the calcium-dependent events involved in oocyte fertilisation have been widely described (Miyazaki, 1991; Yanagimachi, 1988), but the same important role of calcium is evident in the meiotic maturation of oocytes (Homa *et al*., 1993).

All correspondence to: Jiří Rozinek, Department of Veterinary Science, Czech University of Agriculture in Prague, 165 21 Prague 6, Czech Republic. Tel.: +420 224392935. Fax: +420 23438141. e-mail: rozinek@af.czu.cz
[1]Czech University of Agriculture in Prague, Department of Veterinary Science, Prague 6, Czech Republic.
[2]Department of Experimental Medicine, Institute for Clinical and Experimental Medicine, Prague 4, Czech Republic.
[3]Research Institute of Animal Production, Prague 10, Czech Republic.

Meiotic maturation of the oocyte commences at the early stages of oogenesis during the embryonic development of the female. This maturation is later blocked during the early stages of prophase. Resumption of meiosis *in vivo* depends on the stimulation of gonadotropins, but under *in vitro* conditions the release of the oocyte from its follicle alone is often sufficient for meiosis resumption. After the resumption of meiosis, maturation continues through the stages of germinal vesicle breakdown (GVBD), metaphase I, anaphase I, telophase I to the stage of metaphase II, when meiosis is again arrested (Thibault *et al*., 1987; Wassarman, 1988).

The resumption of meiosis is accompanied by typical cytoplasmic oscillations of calcium ions released from intracellular calcium stores and is regulated by calcium-dependent events (Carroll & Swann, 1992; Carroll *et al*., 1994; Lefevre *et al*., 1995). The importance of calcium ions for the resumption of meiosis in the oocytes is emphasised by the fact that chelation of intracellular calcium is able to inhibit the resumption of meiosis in pig (Kaufman & Homa, 1993) and cattle

oocytes (Homa, 1988). The dependence of maturation in pig oocytes on calcium is also supported by the observations that blockage of the influx of calcium ions from extracellular sources resulted in the arrest of meiotic maturation at the stage of metaphase I (Kaufman & Homa, 1993; Petr *et al*., 1997). On the other hand, the release of calcium from intracellular stores at the stage of metaphase I resulted in a significant acceleration of meiotic maturation (Petr *et al*., 1997).

In our previous study (Petr *et al*., 2001) we demonstrated that pig oocytes enclosed in ovarian follicles contain numerous calcium deposits in the nucleus, vacuoles, mitochondria, and on the surface of lipid droplets, but calcium deposits are absent from the endoplasmic reticulum. After oocyte isolation from the follicle, calcium deposits are quickly depleted, and their replenishment occurs gradually during *in vitro* culture of the oocytes.

Intracellular or extracellular calcium could be the source for replenishment of calcium deposits during the *in vitro* culture of oocytes. The aim of the present study was to investigate the role played by extracellular calcium sources in the replenishment of intracellular calcium deposits in pig oocytes maturing *in vitro*. For this purpose we investigated the ultrastructural localisation of calcium deposits after blockage of the calcium channels which mediate the influx of extracellular calcium into the cell.

Materials and methods

Isolation, culture and evaluation of oocytes

Pig ovaries were obtained from a local slaughterhouse from gilts at an unknown stage of the oestrous cycle and transported to the laboratory within 1 h in saline solution (0.9% sodium chloride) at 39 °C. Fully grown oocytes were collected from follicles by aspirating those that were 2–5 mm in diameter with a 20 gauge needle. Only oocytes with compact cumuli were chosen for further studies. Before culture, the oocytes were washed three times in a maturation culture medium.

The oocytes were cultured in a modified M199 medium (GibcoBRL, Life Technologies, Paisley, Scotland) containing sodium bicarbonate (0.039 ml of a 7% solution per millilitre of medium), calcium lactate (0.6 mg/ml), gentamicin (0.025 mg/ml), HEPES (1.5 mg/ml), 13.5 IU eCG: 6.6 IU hCG/ml (P.G.600 Intervet, Boxmeer, Holland) and 10% fetal calf serum (GibcoBRL, Life Technologies, Germany, lot no. 40F2190F).

The oocytes were cultured in 3.5 cm diameter Petri dishes (Nunc, Roskilde, Denmark) containing 3 ml of the culture medium at 39 °C in a mixture of 5% CO_2 in air.

Arrangement of experiments

In the first experiment (experiment 1) we investigated whether and at what concentrations of calcium the channel blocker verapamil influenced *in vitro* maturation in pig oocytes. The oocytes were cultured in a verapamil-supplemented medium (0, 0.01, 0.02 or 0.04 mM) for 48 h.

Experiment 2 was performed to investigate the stage of maturation at which the meiosis of pig oocytes is sensitive to blockage of calcium channels by verapamil. The oocytes were cultured in a verapamil-free medium for 0, 1, 2, 4 or 8 h and were subsequently cultured in a verapamil-supplemented medium (0.02 mM) to complete the total culture time to 48 h.

At the end of the culture, the oocytes were denuded of cumulus cells by repeated pipetting through a narrow glass pipette, mounted on slides, fixed with acetic alcohol (1:3, v/v) for at least 24 h, and stained with 1% orcein. The oocytes were then examined under a phase-contrast microscope.

In a further experiment (experiment 3) we observed changes in the ultrastructural distribution of intracellular calcium deposits in oocytes in which the calcium ion channels had been blocked by verapamil. The oocytes were cultured in a verapamil-supplemented medium (0.02 mM) or a verapamil-free medium for 0, 8, 16, 24, 32 or 40 h and subsequently processed for ultrastructural localisation of intracellular calcium deposits using the combined oxalate–pyroantimonate method.

Ultrastructural localisation of calcium deposits

The oocytes were processed using the combined oxalate–pyroantimonate method (Borgers *et al*., 1981). At the end of the culture, the oocytes were transferred to a primary fixative. They were fixed at 38 °C for 1 h, then for 3 h at room temperature, and finally for 20 h at 4 °C. The primary fixative consisted of 2% glutaraldehyde, 2% formaldehyde, 90 mM potassium oxalate and 1.4% sucrose, pH 7.4. After fixation with a primary fixative, the oocytes were postfixed in 1% osmium tetroxide containing 2% potassium pyroantimonate. Postfixation took 3 h and was carried out at 4 °C. The oocytes were then processed using the standard method for electron microscopy.

Statistical analysis and image analysis

The experiments during which we tested the effects of verapamil on oocyte maturation (experiments 1 and 2) were performed four times. The results were pooled for presentation and evaluated by chi-square analysis (Snedecor & Cochran, 1980). The mean percentage of oocytes reaching a given stage of maturation in all

https://doi.org/10.1017/S0967199403002302 Published online by Cambridge University Press

trials did not vary from the pooled percentage by more than 2.5%. A p value of less than 0.05 was considered significant.

In experiment 3 we evaluated at least five oocytes from each experimental group (at least eight different electron micrographs from each oocyte). These electron micrographs were scanned using an HP ScanJet 6300C scanner, and the digitised data were processed using LUCIA 4.21 software (Laboratory Imaging, Czech Republic). The amount of precipitate was assessed as the percentage of the whole area of the respective cellular compartment. The amount of precipitate was estimated in the nucleus (at the stages preceding germinal vesicle breakdown), vacuoles, mitochondria and cytoplasm. The results were subjected to statistical analysis using an F-test followed by Student's t-test (Snedecor & Cochran, 1980). A p value of less than 0.05 was considered significant.

Results

Effects of verapamil on oocyte maturation

Experiment 1

Oocytes were cultured *in vitro* for 48 h in a medium supplemented with the calcium channel blocker verapamil. Verapamil suppressed oocyte maturation beyond the stage of metaphase I in a dose-dependent manner (Table 1). We chose a concentration of 0.02 mM of verapamil for further experiments because higher concentrations (i.e. 0.04 mM) did not induce a significant increase in the number of oocytes in which maturation was blocked at the stage of metaphase I.

Experiment 2

The critical factor for the effectiveness of verapamil is its addition to the culture medium at the very beginning of *in vitro* maturation of pig oocytes. The oocytes pre-cultured for 2 h in a verapamil-free medium and subsequently cultured with verapamil for 46 h exhibited a significantly higher percentage of oocytes completing their maturation at the stage of metaphase II than those cultured in a verapamil-supplemented medium for 48 h. Oocytes pre-cultured in a verapamil-free medium for more than 8 h did not differ in their maturation from oocytes cultured in a verapamil-free medium for 48 h (Table 2).

Ultrastructural localisation of calcium deposits

Experiment 3

At the beginning of the culture (i.e. immediately after the isolation of oocytes from the follicles) there was only a limited amount of calcium deposits in the nucleus, cytoplasm, mitochondria and vacuoles. During *in vitro* culture the amount of calcium deposits gradually increased. The maximum amount of calcium in the nucleus was observed after 8 h of culture, and it decreased before germinal vesicle breakdown (after 16 h of culture) (Fig. 1), which usually occurred under our culture conditions after 18–20 h of culture. The accumulation of intracellular calcium deposits was observed in other compartments of pig oocytes cultured *in vitro*. The accumulation continued through the stage of metaphase I (24 h of *in vitro* culture) and culminated after 32 h, when the majority of oocytes entered the stage of anaphase I or telophase I. Beyond this time (i.e. after 40 h of *in vitro* culture, when the oocytes had entered the stage of metaphase II), the amount of intracellular calcium deposits decreased.

Under the influence of verapamil, the dynamics of replenishment of intracellular calcium deposits were significantly changed. The amount of deposits peaked in the nucleus after 16 h of *in vitro* culture. The maximum calcium deposits in the mitochondria and cytoplasm occurred at the same time (Fig. 2). There was a significantly higher amount of calcium deposits in verapamil-treated oocytes than in oocytes cultured for the same time in a verapamil-free medium. The replenish-

Table 1 Effects of verapamil on the meiotic maturation of pig oocytes

Concentration of verapamil (mM)	Stage of nuclear maturation (%)				No. of oocytes (n)
	M I	A I	T I	M II	
0	1^a	2^a	2^a	95^a	120
0.01	57^b	0^a	0^a	43^b	120
0.02	72^c	0^a	0^a	28^c	120
0.04	80^c	0^a	0^a	20^c	120

Oocytes were cultured for 48 h with the respective concentration of verapamil, and the stage of meiotic maturation was then assessed

^{a,b,c}Statistically significant differences ($p < 0.05$) between percentages of the respective stage of nuclear maturation (i.e. differences within each column) are indicated by different superscripts.

https://doi.org/10.1017/S0967199403002302 Published online by Cambridge University Press

Table 2 Effects of pre-culture in a verapamil-free medium on the subsequent effects of verapamil on the meiotic maturation of pig oocytes

Preculture time in verapamil free medium (hours)	Stage of nuclear maturation (%)				No. of oocytes (*n*)
	M I	A I	T I	M II	
0	70^{a}	0^{a}	1^{a}	29^{a}	120
1	65^{ab}	0^{a}	0^{a}	35^{ab}	120
2	57^{b}	0^{a}	0^{a}	43^{b}	120
4	33^{c}	4^{a}	0^{a}	63^{c}	120
8	2^{d}	1^{a}	1^{a}	96^{d}	120
48	4^{d}	0^{a}	1^{a}	95^{d}	120

The oocytes were cultured in a verapamil-free medium and then in a medium supplemented with verapamil (0.02 mM). The overall culture time (pre-culture with a verapamil-free medium plus culture with a verapamil-supplemented medium) was 48 h.

^{a,b,c,d}Statistically significant differences ($p < 0.05$) between the percentages of the respective stage of nuclear maturation (i.e. differences within each column) are indicated by different superscripts.

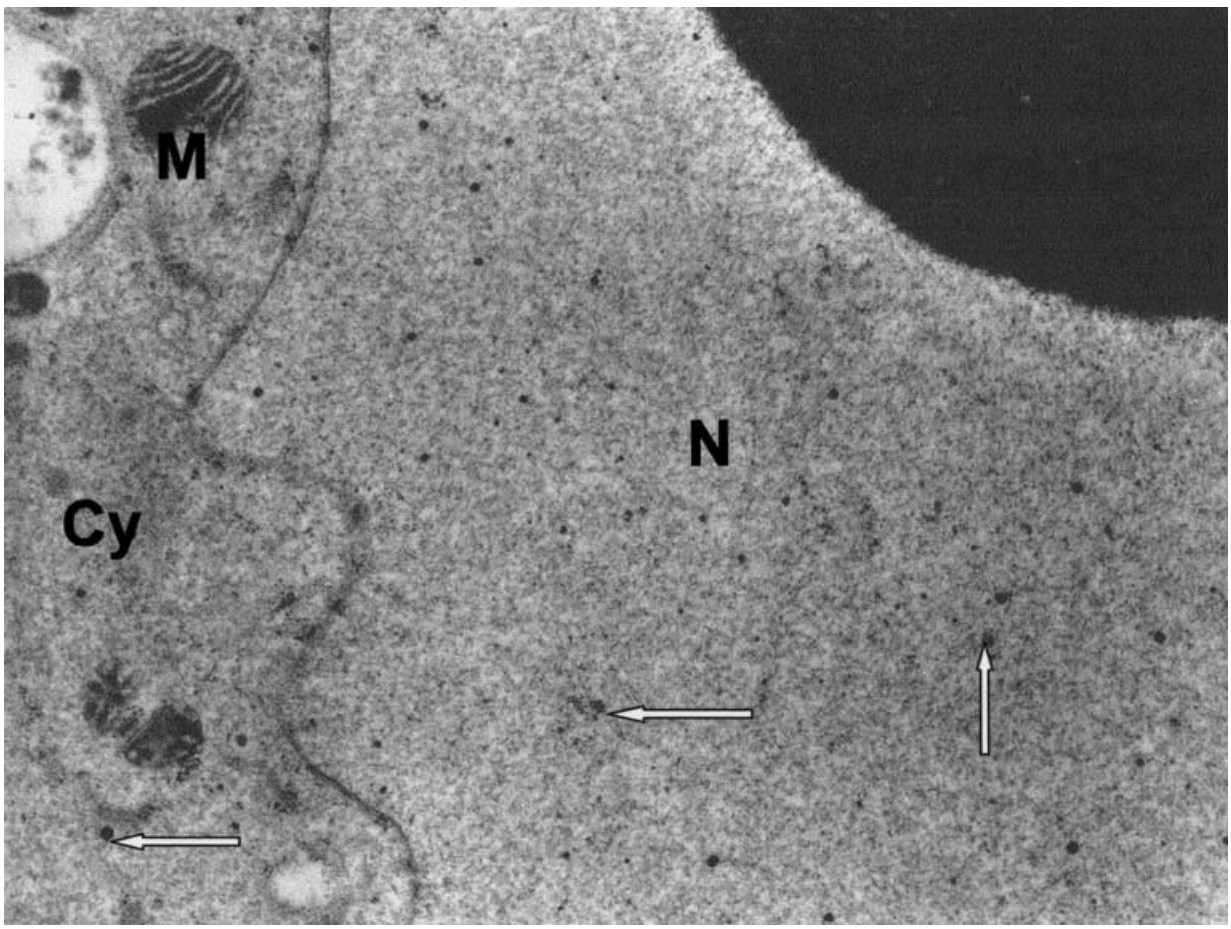

Figure 1 Intracellular calcium deposits in a pig oocyte cultured for 16 h in verapamil-free medium (control). A low level of calcium deposits was present in the oocyte nucleus (N). The level of calcium deposits is even lower in the oocyte cytoplasm (Cy). Mitochondria (M) adjacent to the nuclear membrane did not contain detectable calcium deposits. Magnification ×25 000.

https://doi.org/10.1017/S0967199403002302 Published online by Cambridge University Press

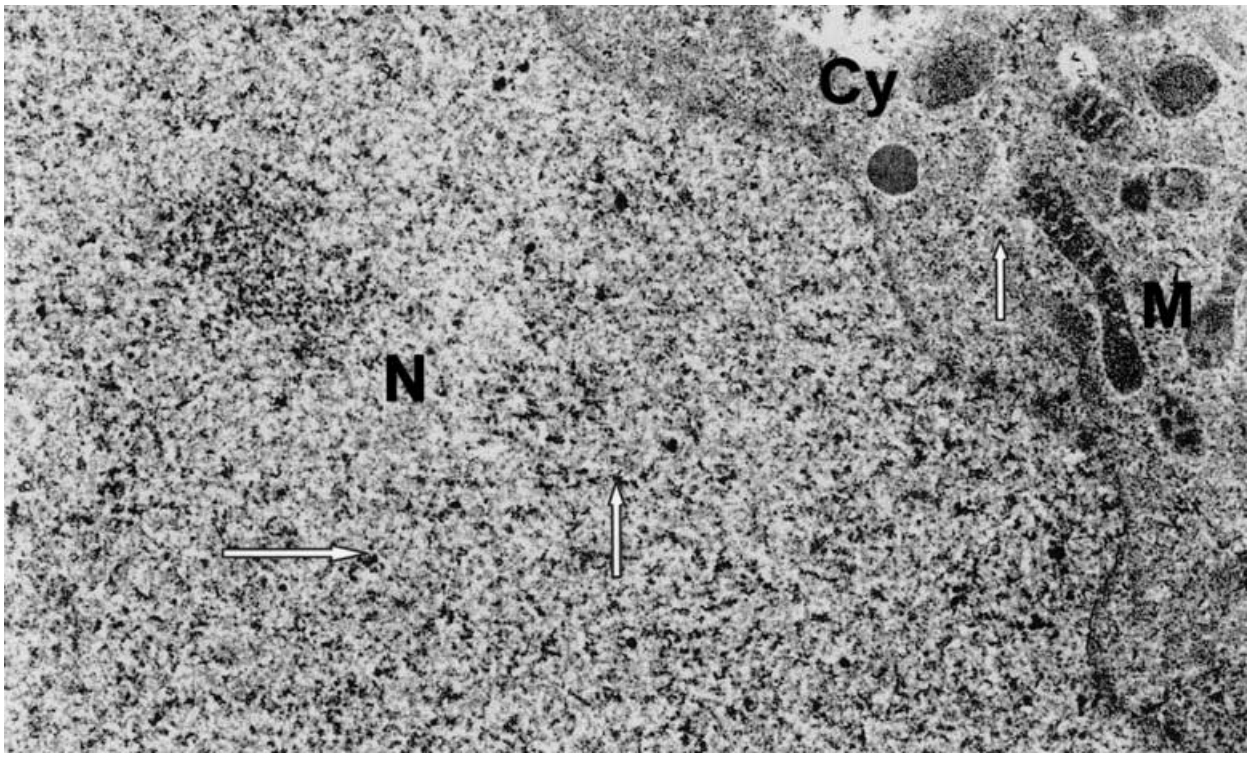

Figure 2 Intracellular calcium deposits in a pig oocyte cultured for 16 hours in verapamil-supplemented medium. Large amounts of calcium were deposited was in the oocyte cytoplasm (Cy), nucleus (N) and mitochondria (M). Magnification ×33 000.

Table 3 Effect of verapamil on calcium deposits in the nucleus of pig oocytes

Time of culture	Control % ± SD	Verapamil % ± SD
0 h	0.69 ± 0.51	
8 h	6.55 ± 4.89[a]	1.64 ± 0.67[b]
16 h	3.14 ± 3.14[a]	7.34 ± 1.49[b]

Oocytes were cultured *in vitro* in a verapamil-free medium (control group) or with 0.02 mM verapamil for 0, 8 or 16 h. The content of intracellular calcium deposits in the nucleus was assessed using a combined oxalate–pyroantimonate method and expressed as a percentage ± standard deviation (SD) of the whole area of the nucleus.
[a,b]Statistically significant differences ($p < 0.05$) between control and verapamil-treated oocytes at given culture times (i.e. within a row) are indicated by different superscripts.

Table 4 Effect of verapamil on calcium deposits in the mitochondria of pig oocytes

Time of culture	Control % ± SD	Verapamil % ± SD
0 h	0.28 ± 0.20	
8 h	0.9 ± 0.46[a]	2.03 ± 1.43[b]
16 h	1.65 ± 0.49[a]	5.02 ± 2.44[b]
24 h	2.06 ± 0.58[a]	3.28 ± 3.37[a]
32 h	2.56 ± 1.09[a]	2.25 ± 1.62[a]
40 h	1.15 ± 0.73[a]	1.17 ± 0.38[a]

Oocytes were cultured *in vitro* in a verapamil-free medium (control group) or with 0.02 mM verapamil for 0, 8, 16, 24, 32 or 40 h. The content of intracellular calcium deposits in the mitochondria was assessed using the combined oxalate–pyroantimonate method and expressed as a percentage ± standard deviation (SD) of the whole area of the mitochondria.
[a,b]Statistically significant differences ($p < 0.05$) between control and verapamil-treated oocytes at given culture times (i.e. within a row) are indicated by different superscripts.

ment of calcium deposits in the vacuoles of verapamil-treated oocytes has similar dynamics to that in oocytes cultured in a verapamil-free medium, with the difference that replenishment in oocytes treated with verapamil for 32 h of culture is significantly greater than in oocytes cultured in a verapamil-free medium for the same length of time.

Differences in the amounts of intracellular deposits in different cellular compartments between verapamil-treated and untreated oocytes are shown in Tables 3, 4, 5 and 6.

https://doi.org/10.1017/S0967199403002302 Published online by Cambridge University Press

Table 5 Effect of verapamil on calcium deposits in the vacuoles of pig oocytes

Time of culture	Control % ± SD	Verapamil % ± SD
0 h	3.20 ± 3.21	
8 h	3.71 ± 1.42[a]	4.15 ± 4.52[a]
16 h	4.94 ± 1.71[a]	4.10 ±2.79[a]
24 h	4.64 ± 1.69[a]	3.62 ± 2.54[a]
32 h	7.52 ± 2.10[a]	5.18 ± 2.75[b]
40 h	5.27 ± 2.90[a]	2.67 ± 1.53[a]

Oocytes were cultured *in vitro* in a verpamil-free medium (control group) or with 0.02 mM verapamil for 0, 8, 16, 24, 32 or 40 h. The content of intracellular calcium deposits in the vacuoles was assessed using the combined oxalate–pyroantimonate method and expressed as a percentage ± standard deviation (SD) of the whole area of the vacuoles.

[a,b]Statistically significant differences ($p < 0.05$) between control and verapamil-treated oocytes at given culture times (i.e. within a row) are indicated by different superscripts.

Table 6 Effect of verapamil on calcium deposits in the cytoplasm of pig oocytes

Time of culture	Control % ± SD	Verapamil % ± SD
0 h	0.41 ± 0.34	
8 h	0.91 ± 0.84[a]	1.02 ± 1.08[a]
16 h	1.68 ± 0.60[a]	3.3 ± 2.60[b]
24 h	1.33 ± 0.42[a]	1.46 ± 1.52[a]
32 h	1.85 ± 0.92[a]	1.23 ± 0.91[b]
40 h	0.46 ± 0.22[a]	1.05 ± 0.49[b]

Oocytes were cultured *in vitro* in a verapamil-free medium (control group) or with 0.02 mM verapamil for 0, 8, 16, 24, 32 or 40 h. The content of intracellular calcium deposits in the cytoplasm was assessed using the combined oxalate–pyroantimonate method and expressed as a percentage ± standard deviation (SD) of the whole area of the cytoplasm.

[a,b]Statistically significant differences ($p < 0.05$) between control and verapamil-treated oocytes at given culture times (i.e. within a row) are indicated by different superscripts.

Discussion

In our study we confirmed the arrest of meiotic maturation in pig oocytes cultured in a medium supplemented with verapamil, a very potent blocker of calcium influx into the cell from extracellular sources (Atlas & Adler, 1981). We also demonstrated changes in the formation of intracellular calcium deposits in pig oocytes cultured *in vitro* under a block of calcium influx from extracellular sources.

The exit of oocytes from the metaphase I stage of meiosis is induced by a decrease in the maturation promoting factor (MPF) (Hashimoto & Kishimoto, 1988), and this decrease also accompanies exit from the stage of metaphase I in pig oocytes (Naito & Toyoda, 1991).

The role of calcium-dependent processes in the regulation of the exit of pig oocytes from the metaphase I stage is indicated by experiments during which the blockage of calcium influx from extracellular sources by verapamil had inhibited the maturation of oocytes beyond the stage of metaphase I and had not allowed the progress of meiosis to the stage of metaphase II (Kaufman & Homa, 1993; Petr *et al*., 1997). On the other hand, the mobilisation of intracellular calcium deposits induced the accelerated exit of pig oocytes from metaphase I (Petr *et al*., 1997). This mobilisation of calcium from intracellular stores was even able to overcome the block of meiotic maturation induced by calcium channel blockage using verapamil (Petr *et al*., 1997). An early start (i.e. the first 8 h) seems to be crucial for the blockage of meiosis of pig oocytes at the stage of metaphase I. When the influx of extracellular calcium is allowed for this crucial interval, the subsequent blockage of calcium influx had no effect on the meiotic maturation of pig oocytes.

The blockage of calcium influx from extracellular sources had a significant effect on the distribution of intracellular calcium deposits. For the detection of these deposits we used the combined oxalate–pyroantimonate method, which has been used effectively for the detection of intracellular calcium stores in various tissues (Borgers *et al*., 1981; Van Reempts *et al*., 1982; Menon *et al*., 1985; Ravindranath *et al*., 1994), human pre-implantation embryos (Sousa *et al*., 1997) and pig oocytes (Petr *et al*., 1997, 1999, 2000, 2001).

A significant amount of calcium ions bound to calcium-binding proteins and detectable using the combined oxalate–pyroantimonate method was present in the follicle-enclosed oocytes (Petr *et al*., 2001). After isolation from the follicle, there was a very rapid depletion of intracellular calcium deposits in the oocytes, and these intracellular stores were gradually replenished during their subsequent *in vitro* culture (Petr *et al*., 2001).

High concentrations of calcium in the extracellular environment represent a natural source for the replenishment of depleted intracellular calcium stores. Empty intracellular calcium stores induce the conditions for calcium entry from extracellular spaces through the process called capacitative calcium entry (Berridge, 1995). Machaty *et al.* (2002) have recently given proof of this mode of replenishment of intracellular calcium stores in matured pig oocytes.

On the other hand, the possibility exists of replenishing intracellular calcium stores through the reuptake of free calcium from the cytoplasm, mediated by calcium-dependent ATPases. Based on our data, we

https://doi.org/10.1017/S0967199403002302 Published online by Cambridge University Press

can conclude that both extracellular and intracellular calcium sources take part in the replenishment of intracellular calcium stores. A significant role of intracellular calcium sources is also clear from our previous study (Petr *et al*., 1997), in which blockage of the reuptake of free calcium ions in pig oocytes at the stage of metaphase I after the inhibition of calcium-dependent ATPases significantly changed the distribution of intracellular calcium deposits.

In this study we observed the replenishment of intracellular calcium stores in maturing pig oocytes even after the blockage of calcium channels. In this case, the intracellular deposits are probably formed from intracellular calcium. The abundance of free calcium ions in mouse oocytes maturing *in vitro* is indicated by oscillating levels of free calcium ions originating from intracellular sources (Carroll & Swann, 1992; Carroll *et al*., 1994; Lefevre *et al*., 1995). The dynamics of replenishment of calcium deposits in pig oocytes cultured *in vitro* is profoundly changed after the blockage of calcium channels when compared with the conditions under which the extracellular sources of calcium are available for the oocyte.

The nuclei of cells contain numerous calcium-binding proteins (Gilchrist *et al*., 1994; Bachs *et al*., 1994), and the nucleus can accumulate calcium at significantly higher levels than can cytoplasm (Himpens *et al*., 1994). In pig oocytes this accumulation of calcium in the nucleus is influenced by the availability of extracellular calcium because there is a significant delay in replenishment of calcium deposits in the nucleus of verapamil-treated oocytes, and GVBD is not preceded by a decrease in calcium deposits in the nucleus.

The presence of numerous calcium deposits in the oocyte mitochondria is not surprising because there is a high internal negative membrane potential on the internal membrane of the mitochondria, and it causes calcium influx into the mitochondria to be energetically 'downhill' (Gunter & Gunter, 1994). But again, the formation of these deposits during *in vitro* maturation of pig oocytes was significantly influenced by the availability of extracellular calcium because the amounts of calcium deposits in the mitochondria of verapamil-treated oocytes were much higher than in the controls.

A remarkable feature is the absence of calcium deposits in the oocyte endoplasmic reticulum, which is thought to be the main source of intracellular calcium (Andreucetti *et al*., 1984; Poenie & Epel, 1987; Han & Nucitelli, 1990; Terasaki & Sardet, 1991; Kline, 2000). On the other hand, Bertout *et al*. (1997) did not find calcium deposits in the endoplasmic reticulum of *Xenopus* oocytes, and Ravindranath *et al*. (1994) did not observe calcium deposits in the endoplasmic reticulum of various mouse testicular cells. One possible explanation for the lack of calcium deposits in the endoplasmic reticulum is that calcium is present there as free calcium ions and could not be detected in this form by the combined oxalate–pyroantimonate method. We cannot exclude the possibility of calcium deposits in the endoplasmic reticulum being easily and quickly mobilised, and their efflux could occur at the earliest stages of specimen preparation.

We may assume that deviations in the formation of calcium deposits in verapamil-treated oocytes can disturb intracellular signalling and cause the arrest of meiosis at the stage of metaphase I. It is known that calcium from the mitochondria is involved in intracellular calcium signalling in *Xenopus* (Jouaville *et al*., 1995) and mouse oocytes (Liu *et al*., 2001). A significant influence of cellular calcium signalling on the activity of MPF was observed in *Xenopus* oocytes (Machaca & Haun, 2002).

Meiotic arrest at the stage of metaphase I in verapamil-treated pig oocytes is not due to a blockage of calcium influx at the crucial interval of exit from metaphase I. An 8 h pre-culture in a verapamil-free medium followed by culture in a verapamil-supplemented medium indicates that allowing calcium influx for the replenishment of intracellular calcium stores at early stages establishes conditions for the appropriate cellular signalling necessary for exit from metaphase I. Several remarkable features were observed in replenishment of intracellular calcium stores during the first 8 h of *in vitro* culture of oocytes, in particular a significant increase in calcium deposits in the nucleus. This increase in nuclear calcium deposits is significantly delayed in verapamil-treated oocytes and can be observed only after 16 h of *in vitro* culture. This early replenishment of nuclear calcium deposits could be one of the critical requirements for subsequent spontaneous exit of the oocyte from metaphase I and the progress of meiosis to metaphase II.

On the basis of our results, we can conclude that influx of extracellular calcium significantly modulates the replenishment of intracellular calcium deposits, which occurs during *in vitro* culture of pig oocytes. Anomalies induced in the pattern of intracellular calcium deposits can disturb conditions suitable for physiological cellular signalling and cause the arrest of meiosis in pig oocytes at the stage of metaphase I.

Acknowledgements

We thank Mrs Lucy Westcott and Mrs Lois Russell for editorial assistance with this manuscript. We would also like to thank the slaughterhouse Jatky Český Brod for kindly providing pig ovaries. Grant sponsors: MZe ČR, grant numbers M02-99-01 and QD 0085.

References

Andreucetti, P., Denis-Donini, S., Burrini, A.G. & Campanella, C. (1984). Calcium ultrastructural localization

https://doi.org/10.1017/S0967199403002302 Published online by Cambridge University Press

in *Xenopus laevis* egg following activation by pricking or by calcium ionophore A 23187. *J. Exp. Zool.* **229**, 295–308.

Atlas, D. & Adler, M. (1981). Alpha-adrenergic antagonists as possible calcium channel inhibitors. *Proc. Natl. Acad. Sci. USA* **78**, 1237–41.

Bachs, O., Agell, N. & Carafoli, E. (1994). Calmodulin and calmodulin-binding proteins in the nucleus. *Cell Calcium* **16**, 289–96.

Berridge, M.J. (1995). Capacitative calcium entry. *Biochem. J.* **312**, 1–11.

Bertout, M., Flament, S., Browaeys-Poly, E. & Vilain, J.-P. (1997). Ultrastructural localization of intracellular calcium stores in *Xenopus* ovarian follicles as revealed by cytochemistry and X-ray microanalysis. *Dev. Growth Differ.* **39**, 249–56.

Borgers, M., Thine, F. & Van Nueten, J. (1981). The subcellular distribution of calcium and effect of calcium-antagonists as evaluated with combined oxalate–pyroantimonate technique. *Acta Histochem.* **24**, 327–33.

Carroll, J. & Swann, K. (1992) Spontaneous cytosolic calcium oscillations driven by inositol triphosphate occurs during *in vitro*. *J. Biol. Chem.* **267**, 11196–201.

Carroll, J., Swann, K., Whittingham, D. & Whitaker, W. (1994). Spatiotemporal dynamics of intracellular $(Ca^{2+})_{(i)}$ oscillations during the growth and meiotic maturation of mouse oocytes. *Development* **120**, 3507–17.

Clapham, D.E. (1995). Calcium signaling. *Cell* **80**, 259–68.

Gilchrist, J.S., Czubryt, M.P. & Pierce, G.N. (1994). Calcium and calcium-binding proteins in the nucleus. *Mol. Cell. Biochem.* **135**, 79–88.

Gunter, K.K. & Gunter, T.E. (1994). Transport of calcium by mitochondria. *J. Bioenerg. Biomembr.* **26**, 471–85.

Han, J.K. & Nuccitelli, R. (1990). Inositol 1,4,5-trisphosphate induced calcium release in the organelle layers of the stratified, intact egg of *Xenopus laevis*. *J. Cell Biol.* **110**, 1103–10.

Hashimoto, N. & Kishimoto, T. (1988). Regulation of meiotic metaphase by a cytoplasmic maturation promoting factor during mouse oocytes maturation. *Dev. Biol.* **126**, 242–52.

Himpens, B., de Smedt, H. & Casteels, R. (1994). Relationship between (Ca^{2+}) changes in nucleus and cytosol. *Cell Calcium* **16**, 239–46.

Homa, S.T. (1988). Effects of cyclic AMP on the spontaneous meiotic maturation of cumulus-free bovine oocytes cultured in chemically defined medium. *J. Exp. Zool.* **248**, 222–31.

Homa, S.T., Carroll, J. & Swan, K. (1993). The role of calcium in mammalian oocyte maturation, and egg activation. *Hum. Reprod.* **8**, 1274–81.

Jouaville, L.S., Chas, F., Holmuhamedov, E.L., Camacho, P. & Lechleiter, J.D. (1995). Synchronization of calcium waves by mitochondrial substrates in *Xenopus laevis* oocytes. *Nature* **377**, 438–41.

Kaufman, M.L. & Homa, S.T. (1993). Defining a role for calcium in the resumption and progression of meiosis in the pig oocytes. *J. Exp. Zool.* **265**, 69–76.

Kline, D. (2000). Attributes and dynamics of the endoplasmic reticulum in mammalian eggs. *Curr. Topics Dev. Biol.* **50**, 125–54.

Lefevre, B., Pesty, A. & Testart, J. (1995). Cytoplasmic and nucleic calcium oscillations in immature mouse oocytes: evidence of wave polarization by confocal imaging. *Exp. Cell Res.* **218**, 166–73.

Liu, L., Hammar, K., Smith, P.J.S., Inoue, S. & Keefe, D.L. (2001). Mitochondrial modulation of calcium signalling at the initiation of development. *Cell Calcium* **30**, 423–33.

Machaca, K. & Haun, S. (2002). Induction of maturation-promoting factor during *Xenopus* oocyte maturation uncouples Ca^{2+} store depletion from store-operated Ca^{2+} entry. *J. Cell Biol.* **156**, 75–85.

Machaty, Z., Ramsoondar, J.J., Bonk, A.J., Bondioli, K.R. & Prather, R.S. (2002). Capacitative calcium entry mechanism in porcine oocytes. *Biol. Reprod.* **66**, 667–74.

Menon, G.K., Grayson, S. & Elias, P.M. (1985). Ionic calcium reservoirs in mammalian epidermis: ultrastructural localisation by ion-capture cytochemistry. *J. Invest. Dermatol.* **84**, 508–12.

Miyazaki, S. (1991). Repetitive calcium transients in hamster oocytes. *Cell Calcium* **12**, 205–16.

Naito, K. & Toyoda, Y. (1991). Fluctuation of histone H1 kinase activity during meiotic maturation in porcine oocytes. *J. Reprod. Fertil.* **93**, 467–73.

Petr, J., Rozinek, J., Hruban, V., Jílek, F., Sedmíková, M., Vaňourková, Z., Němeček Z. (2001). Ultrastructural localization of calcium deposits during *in vitro* culture of pig oocytes. *Mol. Reprod. Dev.* **58**, 196–204.

Petr, J., Rozinek, J. & Jílek, F. (1997). Cyclopiazonic acid induces accelerated progress of meiosis in pig oocytes. *Zygote* **5**, 193–205.

Petr, J., Rozinek, J., Jílek, F. & Urbánková, D. (2000). Activation of porcine oocytes using cyclopiazonic acid, an inhibitor of calcium-dependent ATPases. *J. Exp. Zool.* **287**, 304–15.

Petr, J., Rozinek, J., Vaňourková, Z. & Jílek, F. (1999). Cyclopiazonic acid, an inhibitor of calcium-dependent ATPases, induces exit from metaphase I arrest in growing pig oocytes. *Reprod. Fertil. Dev.* **11**, 235–46.

Poenie, M. & Epel, D. (1987). Ultrastructural localization of intracellular calcium stores by a new cytochemical method. *J. Histochem. Cytochem.* **35**, 939–59.

Ravindranath, N., Papadopoulos, V., Vornberger, W., Zitzmann, D. & Dym, M. (1994). Ultrastructural distribution of calcium in the rat testis. *Biol. Reprod.* **51**, 50–62.

Snedecor, G.W. & Cochran, W.G. (1980). *Statistical Methods*, 7th edn. Iowa: Iowa State University Press.

Sousa, M., Barros, A., Silva, J. & Tesarik, J. (1997). Developmental changes in calcium content of ultrastructurally distinct subcellular compartments of preimplantation human embryos. *Mol. Hum. Reprod.* **3**, 83–90.

Terasaki, M. & Sardet, C. (1991). Demonstration of calcium uptake and release by sea urchin egg cortical endoplasmic reticulum. *J. Cell Biol.* **115**, 1031–7.

Thibault, C., Szollosi, D. & Gerard, M. (1987). Mammalian oocyte maturation. *Reprod. Nutr. Dev.* **27**, 865–96.

Van Reempts, J., Borgers, M. & Offner, F. (1982). Ultrastructural localization of calcium in the rat retina with a combined oxalate–pyroantimonate technique. *Histochem. J.* **14**, 517–22.

Wassarman, P.M. (1988). The mammalian ovum. In *The Physiology of Reproduction* (ed. E. Knobil & J. Neill), pp. 69–102. New York: Raven Press.

Yanagimachi, R. (1988). Mammalian fertilization. In: *The Physiology of Reproduction* (ed. E. Knobil & J. Neill), pp. 135–85. New York: Raven Press.