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Defective calcium release during *in vitro* fertilization of maturing oocytes of LT/Sv mice

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ABSTRACT Oocytes of LT/Sv mice have anomalous cytoplasmic and nuclear maturation. Here, we show that in contrast to the oocytes of wild-type mice, a significant fraction of LT/Sv oocytes remains arrested at the metaphase of the first meiotic division and is unable to undergo sperm-induced activation when fertilized 15 hours after the resumption of meiosis. We also show that LT/Sv oocytes experimentally induced to resume meiosis and to reach metaphase II are unable to undergo activation in response to sperm penetration. However, the ability for sperm-induced activation developed during prolonged *in vitro* culture. Both types of LT/Sv oocytes, i.e. metaphase I and those that were experimentally induced to reach metaphase II, underwent activation when they were fertilized 21 hours after germinal vesicle breakdown (GVBD). Thus, the ability of LT/Sv oocytes to become activated by sperm depends on cytoplasmic maturation rather than on nuclear maturation i.e. on the progression of meiotic division. We also show that sperm penetration induces fewer Ca^{2+} transients in LT/Sv oocytes than in control wild-type oocytes. In addition, we found that the levels of mRNA encoding different isoforms of protein kinase C (α, δ and ζ), that are involved in meiotic maturation and signal transduction during fertilization, differed between metaphase I LT/Sv oocytes which cannot be activated by sperm, and those which are able to undergo activation after fertilization. However, no significant differences between these oocytes were found at the level of mRNA encoding IP_3 receptors which participate in calcium release during oocyte fertilization.

KEY WORDS: *LT/Sv, meiosis, in vitro fertilization, activation, calcium oscillations*

Introduction

Progression of mouse oocytes through meiotic cycle, commonly described as "meiotic maturation", involves both nuclear and cytoplasmic changes (Eppig, 1996). Nuclear maturation refers to the progression of the oocyte from prophase I arrest to metaphase II arrest and involves the breakdown of nuclear envelope, chromatin condensation and the formation of metaphase I and, after first meiotic division, metaphase II spindle (Eppig, 1996). Cytoplasmic maturation consists of a number of changes that prepare the oocyte for sperm-induced activation, i.e. enable it to remodel the sperm nucleus and to support early stages of embryonic development (Carroll *et al.,* 1996; Eppig, 1996). These changes include an increase in the level of intracytoplasmic glutathione, which is necessary for decondensation of sperm nucleus after fertilization (Perreault *et al.,* 1988), the increase in the number of type I inositol 1,4,5-trisphosphate receptors (IP_3R-1), and the increase in the level of Ca^{2+} stored in endoplasmic reticulum (Tombes *et al.,* 1992; Carroll *et al.,* 1994; Jones *et al.,* 1995; Mehlmann *et al.,* 1996; Parrington *et al.,* 1998; Fissore *et al.,* 1999; Xu *et al.,* 2003). Some of these changes are crucial for the ability of oocyte to generate Ca^{2+} oscillations in response to sperm penetration that are necessary and sufficient to induce oocyte activation and progression through the first embryonic cell cycle (Ducibella *et al.,* 2006). Mechanism responsible for oocyte activation via repetitive calcium signals involves Ca^{2+}/calmodulin-

Abbreviations used in this paper: CSF, cytostatic factor; GVBD, germinal vesicle breakdown; IP3R, inositol 1,4,5-trisphosphate receptor; MPF, M-phase promoting factor; PKC, protein kinase C.

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dependent protein kinase II (CaMKII) pathway. Activation of this kinase is crucial for the inactivation of two factors, M-phase promoting factor (MPF) and cytostatic factor (CSF; Schmidt *et al.,* 2006). MPF is a major M-phase regulator consisting of protein kinase CDK1 and cyclin B. In metaphase II-arrested oocytes, its level is sustained due to the activity of CSF (Jones, 2004; Schmidt *et al.,* 2006) whose key component, endogenous meiotic inhibitor 2 (Emi2), prevents the degradation of cyclin B by inhibiting Anaphase Promoting Complex (APC; Madgwick *et al.,* 2006; Shoji *et al.,* 2006). Activation of CaMKII leads to the degradation of Emi2 and release of APC from CSF arrest, thus allowing the completion of meiotic division.

Oocytes of LT/Sv mouse strain are an example of the abnormal regulation of meiotic maturation. Many of these oocytes are unable to complete first meiotic division and become arrested at metaphase I (MI) stage (Kaufman and Howlett, 1986; O'Neill and Kaufman, 1987; Maleszewski and Yanagimachi, 1995; Ciemerych and Kubiak, 1998). Importantly, MI-arrested oocytes localized within the ovary can undergo spontaneous parthenogenetic activation. Formation of parthenogenetic embryos, which develop chaotically may eventually result in the ovarian teratomas or teratocarcinomas (Stevens and Varnum, 1974; Eppig *et al.,* 1977; Artzt *et al.,* 1987). Ovulated LT/Sv metaphase I oocytes can undergo spontaneous activation and develop into diploid parthenogenetic embryos that are able to reach the blastocyst stage and implant within the uterus (Eppig, 1978). Several research groups reported that fertilization of MI-arrested LT/Sv oocytes triggered their activation and resulted in the development of digynic triploid conceptuses (Kaufman and Speirs, 1987; O'Neill and Kaufman, 1987; Speirs and Kaufman, 1990; West *et al.,* 1993). However, we demonstrated that majority of metaphase I-arrested LT/Sv oocytes were not competent to be activated by sperm and remained arrested at metaphase I (Maleszewski and Yanagimachi, 1995; Ciemerych and Kubiak, 1998). In such 'sperm-carrying' metaphase I LT/Sv oocytes spontaneous parthenogenetic activation induces formation of a male pronucleus (Ciemerych and Kubiak, 1998).

Oocytes isolated from ovaries of sexually immature wild-type mice [F_1 (C57BL/6J x SJL)] are another example of premature termination of meiotic maturation. These oocytes begin maturation, but are unable to reach metaphase II stage and, similarly to LT/Sv oocytes, become arrested at metaphase I (Eppig *et al.,* 1994). However, in contrast to MI-arrested LT/Sv oocytes, they can be activated by sperm or parthenogenetic agents with the same frequency as ovulated, metaphase II-arrested oocytes (Eppig *et al.,* 1994). These data indicate that the ability of mouse oocytes to be activated depends on completion of cytoplasmic but not nuclear maturation (Eppig *et al.,* 1994). Therefore, it is possible that inability of MI-arrested LT/Sv oocytes to be activated by sperm penetration is related to their abnormal cytoplasmic maturation.

Previously published studies described the behavior of '*in vitro* cultured MI-arrested LT/Sv oocytes that were fertilized within 15 h after GVBD (Maleszewski and Yanagimachi, 1995; Ciemerych and Kubiak, 1998). Behavior of MI-arrested LT/Sv oocytes that were fertilized later than 15 h after GVBD has never been documented. Therefore, it remained unknown whether these oocytes can acquire the cytoplasmic maturity to become activated by sperm. To answer this question we compared behavior of metaphase I LT/Sv oocytes, which were fertilized 15 and 21 h after GVBD. Moreover, in order to examine whether completion of nuclear maturation influence the sperm-induced activation, we compared the frequency of activation of LT/Sv oocytes arrested at metaphase I and age-matched LT/Sv oocytes that were experimentally induced to complete the first meiotic division and reached metaphase II. Since prolonged *in vitro* culture of metaphase II-arrested wild-type oocytes leads to the decrease in MPF activity and therefore facilitates their activation (Fissore *et al.,* 2002), we asked whether the differences in the behavior of LT/Sv oocytes studied at different time points after GVBD could be explained by the changes of MPF activity. Finally, as it is known that oocyte activation requires calcium-dependent degradation of cyclin B (Hyslop *et al.,* 2004), we followed the Ca^{2+} oscillations in age-matched wild-type and LT/Sv oocytes during fertilization. We also analyzed the expression of type I inositol 1,4,5-trisphosphate receptors (IP_3R-1) and α, δ and ζ isoforms of protein kinase C (PKC) which are involved in regulation of signaling pathways crucial for the meiotic maturation and/or activation of oocytes (Halet, 2004; Lee *et al.,* 2006).

Results

Delayed maturation of in vitro *cultured LT/Sv oocytes*

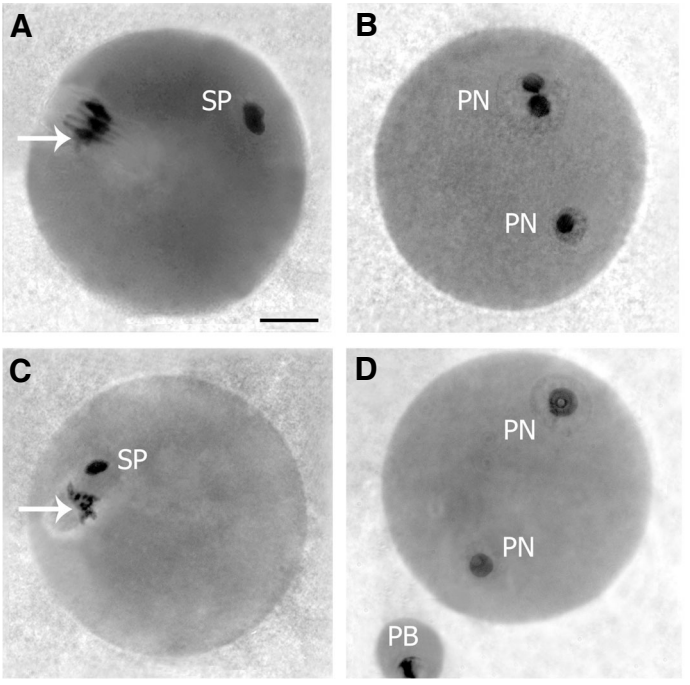
Ninety one percent (763/843) of LT/Sv oocytes isolated at GV stage resumed meiosis i.e. underwent GVBD during first 2 hours of *in vitro* culture. Only these oocytes were selected for further studies. Ten, 15 or 21 h after GVBD the oocytes were scored for the presence of the first polar body, a sign that the first meiotic division was completed and oocytes reached metaphase II. Control wild-type oocytes were isolated from ovarian follicles and cultured *in vitro* simultaneously with LT/Sv oocytes. Ninety percent (235/260) of wild-type oocytes underwent GVBD during the first two hours after isolation. Ten hours after GVBD 79% (352/447) of LT/Sv oocytes were still at metaphase I while 97% (228/235) of wild-type oocytes completed first meiotic division and reached metaphase II. Fifteen and 21 hours after GVBD 50.5% (101/200) and 42.3% (118/279) of LT/Sv oocytes were arrested at MI, respectively. These results agree with previous studies showing that significant proportion of *in vitro* maturing LT/Sv oocytes did not complete the first meiotic division and remained arrested at metaphase I stage (Albertini and Eppig, 1995; Eppig *et al.,* 1996; Ciemerych and Kubiak, 1998). The remaining LT/Sv oocytes either progressed to metaphase II stage or became activated parthenogenetically. Activation rate was 10.1% (10/99) and 16.1% (26/161) for oocytes

TABLE 1

REACTION OF METAPHASE I-ARRESTED LT/SV OOCYTES TO FERTILIZATION 15 OR 21 H AFTER GVBD

		Hours after GVBD at the moment of fixation			
		21		27	
		fertilized oocytes	control oocytes	fertilized oocytes	control oocytes
Oocyte stage at fixation	Metaphase I	34/44 (77%)	27/31 (87%)	2/38 (5%)	32/33 (97%)
	Metaphase II	4/44 (9%)	3/31 (10%)	0/38	0/33
	Interphase	6/44 (14%)	1/31 (3%)	36/38 (95%)	1/33 (3%)

Oocytes were fixed 21 or 27 h after germinal vesicle breakdown (GVBD; 6 h after fertilization)



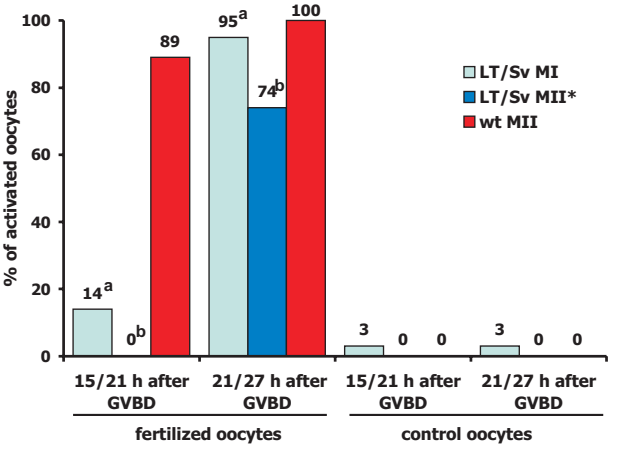


Fig. 1 (Left). Morphology of LT/Sv oocytes fertilized at metaphase I (A, B) and metaphase II* (C, D) 15 or 21 h after germinal vesicle breakdown (GVBD), and fixed 6 h after fertilization (21 and 27 h after GVBD). **(A)** *Oocyte fertilized 15 h and fixed 21 h after GVBD that remained arrested at metaphase I and contained condensed sperm chromatin.* **(B)** *Metaphase I oocyte fertilized 21 h and fixed 27 h after GVBD that completed first meiotic division and formed a one-cell embryo with two pronuclei.* **(C)** *Oocyte fertilized 15 h and fixed 21 h after GVBD that remained arrested at metaphase II* with condensed sperm chromatin localized on the metaphase spindle.* **(D)** *Metaphase II* oocyte fertilized 21 h and fixed 27 h after GVBD that completed second meiotic division and formed one-cell embryo with female (smaller, located close to the polar body) and male (bigger) pronucleus. Arrow indicates metaphase chromosomes. SP, condensed sperm chromatin; PN, pronucleus; PB, polar body. Bar represents 20 μm.*

Fig. 2 (Right). Reaction of metaphase I-arrested and metaphase II*-arrested LT/Sv oocytes to fertilization. *Oocytes were fertilized 15 or 21 h after GVBD and fixed 21 or 27 h after GVBD. Graph represents the percentage of oocytes that become activated as a result of sperm penetration (fertilized) or spontaneously during* in vitro *culture (control). Columns marked with letters are significantly different (P < 0.05)*

scored at 15h and 21 h after GVBD, respectively. Both metaphase II and parthenogenetically activated oocytes were excluded form further analyses. Thus, the number of metaphase I-arrested LT/Sv oocytes decreased between 10 and 21 h after GVBD reflecting the previously described phenomenon that, in contrast to wild-type oocytes that complete first meiotic division 8-9 hours after GVBD, in LT/Sv oocytes the completion of the first meiotic division is severely delayed (Ciemerych and Kubiak, 1998).

Ability to be activated by sperm develops during prolonged in vitro *culture of metaphase I LT/Sv oocytes*

Previous studies showed that MI-arrested LT/Sv oocytes which were fertilized 15 h after GVBD did not undergo activation in response to sperm penetration (Maleszewski and Yanagimachi, 1995; Ciemerych and Kubiak, 1998). Only LT/Sv oocytes that reached MII were able to become activated by sperm (Maleszewski and Yanagimachi, 1995). We showed previously that the progression through meiotic division is delayed in significant fraction of LT/Sv oocytes (Ciemerych and Kubiak, 1998). It is possible that other cellular processes are also defective or delayed during maturation of LT/Sv oocytes. Therefore, we decided to determine whether the development of the ability to become activated by sperm is also delayed in LT/Sv oocytes.

In vitro maturing MI LT/Sv oocytes selected 15 and 21 h after GVBD (n=75 and n=71, respectively) were either inseminated (experimental group) or cultured without insemination (control group; Table 1). Oocytes that by the time of selection for the insemination reached MII or became parthenogenetically activated were excluded from further analyses. Experimental and control oocytes were then cultured for additional 6 h and fixed 21 or 27 h after GVBD, respectively. Seventy seven percent of MI LT/Sv oocytes fertilized 15 h after GVBD remained arrested at MI at the time of fixation i.e. 21 h after GVBD (Fig. 1A; Table 1). Only 23% of them underwent first polar body extrusion and either reached the metaphase II stage (9%) or become activated and formed interphase pronuclei (14%) (Fig. 2; Table 1). In comparison, high percentage, i.e. 95% ($P < 0.05$), of LT/Sv oocytes fertilized 21 h after GVBD underwent the first polar body extrusion and formed interphase pronuclei (Fig. 1B; Fig. 2; Table 1). The majority of control metaphase I LT/Sv oocytes that were cultured simultaneously with fertilized oocytes remained arrested at M-phase, proving that culture conditions alone did not cause the oocyte activation. When examined 21 and 27 h after GVBD only 3% of control oocytes, that were not inseminated, underwent spontaneous activation and contained interphase nuclei (Fig. 2, Table 1). Therefore, we concluded that the difference in frequency of activated LT/Sv oocytes fertilized 15 or 21 h after GVBD reflected the development of the ability to respond to fertilization rather than a side effect of a prolonged *in vitro* culture.

Cytoplasmic, not nuclear, maturation of LT/Sv oocytes is required for development of their ability to be activated by spermatozoa

Hirao and Eppig (1999) showed that the incubation of MI-arrested LT/Sv oocytes in the presence of an unspecific kinase inhibitor 6-DMAP induces completion of the first meiotic division.

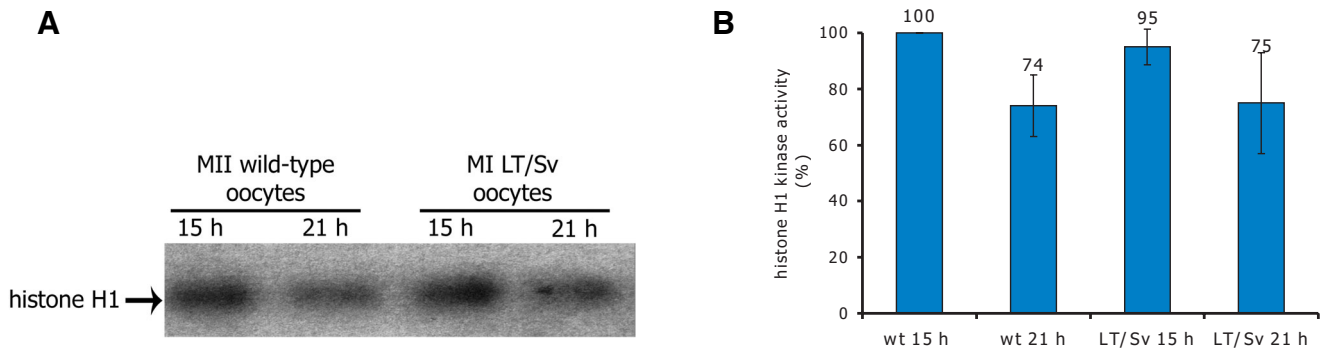


Fig. 3. Histone H1 kinase activity in metaphase I-arrested LT/Sv and metaphase II-arrested wild-type oocytes. (A) *Autoradiograph of histone H1 kinase assay performed on wild-type and LT/Sv oocytes collected 15 and 21 h after germinal vesicle breakdown (GVBD).* **(B)** *The graph shows histone H1 kinase activity (represented by the intensity of histone H1 phosphorylation bands) in metaphase I LT/Sv and metaphase II wild-type oocytes analyzed 15 and 21 h after GVBD.*

We modified this experiment and before the exposure to 6-DAMP we pretreated the LT/Sv oocytes that persisted in MI for 10 h after GVBD with a brief (5 min) incubation in calcium ionophore A23187, an agent inducing Ca^{2+} release (see Material and Methods). Among these oocytes 53% (186/352) completed first meiotic division and reached metaphase II stage (hereinafter called metaphase II*, MII*), i.e. extruded the first polar body but did not form interphase nucleus within 90 min of culture. These oocytes were cultured for another 3 or 9 h after completion of the first meiotic division, i.e. for total 15 h (n=84) or 21 h (n=62) after GVBD respectively. Thus, MII* oocytes were cultured for the same period after GVBD as MI-arrested LT/Sv oocytes described in previous paragraph. Subsequently, these oocytes were either fertilized or cultured without any manipulations, and then fixed after additional 6 hours of culture i.e. 21 or 27 h after GVBD, respectively. Control, age-matched wild-type MII-arrested oocytes were fertilized and fixed at the same time points as LT/Sv oocytes. All of the wild-type oocytes fertilized 21 h after GVBD (n=21) and 88.8% (24/27) of oocytes fertilized 15 h after GVBD became activated (Fig. 2). None of MII* LT/Sv oocytes (n=49) that were fertilized 15 h and fixed 21 h after GVBD underwent activation in response to a sperm penetration i.e. neither extruded the second polar body nor formed pronuclei (Fig. 1C; Fig. 2). In contrast, 74% (31/42; $P < 0.05$) of MII* LT/Sv oocytes fertilized 21 h and fixed 27 h after GVBD became activated (Fig. 1D; Fig. 2). None of control MII* LT/Sv oocytes (n=35 and n=20, fixed 21 and 27 h after GVBD, respectively) that were not subjected to *in vitro* fertilization underwent spontaneous activation (Fig. 2), indicating that the activation of fertilized oocytes was triggered solely by a sperm penetration.

Thus, LT/Sv oocytes which were experimentally induced to reach MII* acquired the ability to be activated by sperm penetration at the same time after resumption of meiosis (between 15 and 21 h after GVBD) as MI-arrested LT/Sv oocytes. Therefore, we concluded that the ability of LT/Sv oocytes to become activated by sperm depends on status of their cytoplasmic maturation rather than on the progression of meiotic division.

Decrease in MPF activity accompanies the development of the ability of MI-arrested LT/Sv oocytes to undergo sperm-induced activation

Metaphase II arrest of oocytes is maintained by CSF that prevents cyclin B degradation and inactivation of MPF (Jones, 2004; Schmidt *et al.,* 2006). Fissore *et al.* (2002) showed that the MPF activity decreases during prolonged *in vitro* culture of mouse oocytes leading to the increased incidence of spontaneous activation or susceptibility to induced activation. However, Hampl and Eppig (1995) showed that the rise in MPF activity accompanied metaphase I arrest of oocytes of sexually immature wild-type mice and was also observed in metaphase I LT/Sv oocytes cultured for 14 h after GVBD. It is possible that the increased susceptibility of metaphase I- arrested LT/Sv oocytes, cultured for prolonged time after GVBD, for sperm-induced or parthenogenetic activation is also caused by the decrease in the MPF activity. Thus, we decided to determine whether the change in the MPF activity parallels the increase in frequency of sperm-induced activation of LT/Sv oocytes fertilized 21 h after GVBD.

Histone H1 kinase activity (the biochemical indicator of MPF activity) was assessed in MI-arrested LT/Sv oocytes collected 15 and 21 h after GVBD. Since wild-type oocytes rarely arrest at MI, the MPF activity detected in wild-type MII oocytes collected either 15 or 21 h after GVBD were used as a standard for the activity assayed in MI LT/Sv oocytes (Fig. 3; see Material and Methods). We did not detect any significant differences in activity of kinase of histone H1 between MI-arrested LT/Sv oocytes collected 15 after GVBD and the age-matched wild-type MII oocytes (Fig. 3B; $P > 0.05$). Similarly, there was no difference in histone H1 kinase activity between MI LT/Sv and MII wild-type oocytes assayed 21 h after GVBD (Fig. 3B; $P > 0.05$). We noticed the slight decline of histone H1 kinase activity in both MI LT/Sv and MII wild-type oocytes collected 21 h after GVBD as compared to activity assayed in oocytes collected 15 h after GVBD, but the difference was not statistically significant (Fig. 3 A,B; $P > 0.05$). Therefore, we cannot exclude that the decrease in the MPF activity observed in MI LT/Sv oocytes 21 h after GVBD may facilitate their activation after fertilization, however, this does not seem to be a crucial factor in sperm-induced activation of these oocytes.

Impaired Ca^{2+} oscillations in fertilized metaphase I LT/Sv oocytes

Since the activation of oocytes arrested either at metaphase I or metaphase II requires calcium-dependent degradation of cyclin B, a regulatory subunit of MPF (Hyslop *et al.,* 2004), we decided to study the pattern of Ca^{2+} oscillations during *in vitro* fertilization. Ca^{2+} response of LT/Sv oocytes fertilized at MI stage was com-

pared to MII wild-type oocytes that generate multiple Ca^{2+} oscillations after sperm penetration (Cuthbertson and Cobbold, 1985; Kline and Kline, 1992). Both types of oocytes were fertilized either 15 or 21 h after GVBD. As expected, 91.6% (11/12) of MII wild-type oocytes fertilized 15 h and 100% (9/9) of MII wild-type oocytes fertilized 21 h after GVBD generated 3 to 8 Ca^{2+} transients within 90 min after sperm penetration (Fig. 4 A,B). Subsequently, these oocytes become activated. In contrast, during the same period of time only 9.5% (2/19; $P < 0.05$) of MI LT/Sv oocytes fertilized 15 h after GVBD had more than two Ca^{2+} transients. The majority of these oocytes (90.5%; 17/19) generated only one or two Ca^{2+} transients (Fig. 4C) and remained arrested at MI. Similarly, one or two Ca^{2+} transients occurred in 78.5% (11/14) of MI LT/Sv oocytes inseminated 21 h after GVBD (Fig. 4D). More than two Ca^{2+} transients occurred only in 21.5% (3/14) of such oocytes, which was significantly less ($P<0.05$) than in age-matched wild-type oocytes. Majority of oocytes with two or more Ca^{2+} transients become activated. Thus, we concluded that the generation of Ca^{2+} oscillations is impaired in LT/Sv oocytes fertilized at metaphase I stage.

Moreover, we observed similar impaired pattern of calcium transients in LT/Sv oocytes that spontaneously completed the first meiotic division, reached metaphase II stage and then were fertilized at 15 or 21 h after GVBD. The majority of these oocytes generated only one or two Ca^{2+} transients when fertilized 15 or 21 h after GVBD (61.5%, 8/13 and 70%, 7/10, respectively; Fig. 4 E,F). Only 38.5% (5/13) of MII LT/Sv oocytes fertilized 15 h after GVBD and 30% (3/10) of oocytes fertilized 21 h after GVBD had more than two Ca^{2+} transients, and this number was significantly lower then in age-matched wild-type oocytes (compare Fig. 4 A,B; $P < 0.05$). Since metaphase II oocytes, similarly to metaphase I LT/Sv oocytes fertilized 21 h after GVBD, became activated, we concluded that impairment in Ca^{2+} oscillations does not influence the ability of these oocytes to be activated by sperm. Moreover, the fact that 21 h after GVBD the LT/Sv oocytes are unable to generate long-lasting Ca^{2+} oscillations indicates that their cytoplasmic maturation is incomplete.

Expression of IP_3R-1, PKCα, PKCδ and PKCζ in metaphase I LT/Sv oocytes

Meiotic maturation and activation of mouse oocytes are regulated by several isoforms of protein kinase C (PKC) (reviewed by Halet, 2004). Ca^{2+} oscillations depend on calcium release from endoplasmic reticulum that occurs after binding of inositol 1,4,5-trisphosphate (IP_3) to type 1 IP_3 receptors (IP_3R-1). Because our experiments showed that the fertilization of LT/Sv oocytes did not result in the repetitive Ca^{2+} oscillations we focused on the factors involved in Ca^{2+} signaling.

Using RT-PCR we compared the levels of mRNAs encoding IP_3R-1 and different PKC isoforms between MI-arrested LT/Sv oocytes and age-matched, MII–arrested wild-type oocytes collected at 15 h or 21 h after GVBD (Fig. 5 A,B). Since it is known that the translation of given mRNA correlates with its polyadenylation status, only polyadenylated forms of mRNA were isolated from oocytes (Piccioni *et al.,* 2005; Cui and Kim, 2007). The levels of mRNA detected in MI LT/Sv oocytes were calculated as a percentage of mRNA level (denoted arbitrally as 100%) in age-matched MII wild-type oocytes. In each of three experiments we obtained similar results, which suggests that the differences in the expression of analyzed genes did not result from the errors in experimental procedure (see Supplementary Material, Table S1).

Analysis of the level of IP_3R-1 mRNA revealed no significant differences between LT/Sv oocytes analyzed 15 and 21 h after GVBD ($P > 0.05$) and was similar to that of age-matched wild-type oocytes (134% and 99% of wild-type levels, respectively; $P > 0.05$) (Fig. 5A). In contrast, the level of mRNAs encoding PKCα and PKCδ detected in MI LT/Sv oocytes collected 15 h after GVBD was higher than in age-matched

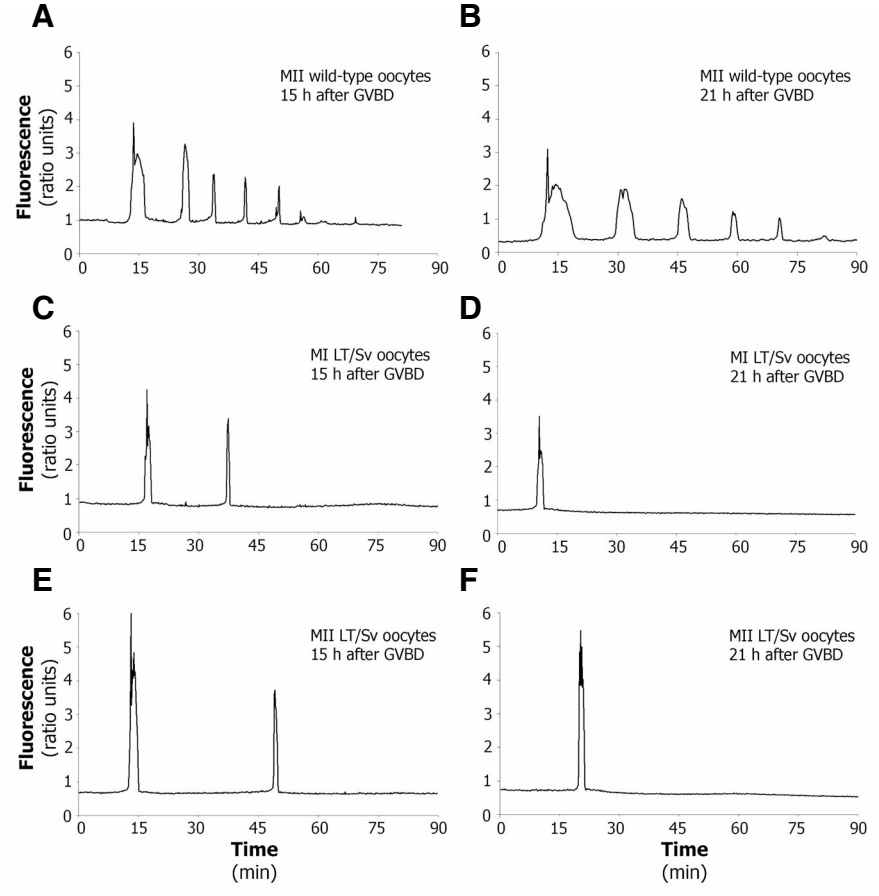
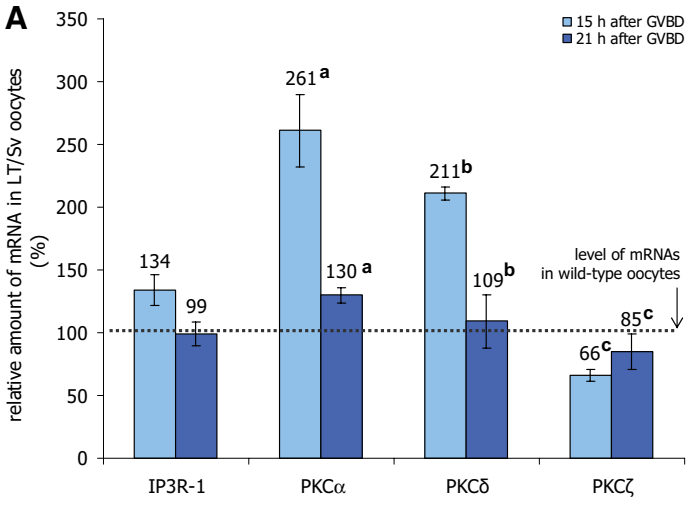


Fig. 4. Ca^{2+} oscillations in metaphase I-arrested LT/Sv and metaphase II-arrested wild-type oocytes after fertilization. *Measurements of Ca^{2+} oscillations in metaphase I LT/Sv and metaphase II wild-type oocytes fertilized 15 or 21 h after GVBD. Oscillations were measured within 90 min after fertilization.* **(A)** *Metaphase II wild-type oocyte fertilized 15 h after GVBD;* **(B)** *metaphase II wild-type oocyte fertilized 21 h after GVBD;* **(C)** *metaphase I LT/Sv oocyte fertilized 15 h after GVBD;* **(D)** *metaphase I LT/Sv oocyte fertilized 21 h after GVBD;* **(E)** *metaphase II LT/Sv oocyte fertilized 15 h after GVBD;* **(F)** *metaphase II LT/Sv oocyte fertilized 21 h after GVBD. Ca^{2+} levels are presented as the ratio of fura 2–AM fluorescence at 340 and 380 nm.*



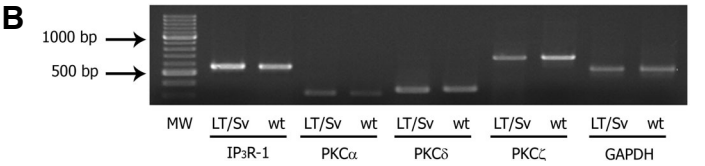


Fig. 5. mRNA level of *IP$_3$R-1, PKCα, PKCδ* and *PKCζ* in metaphase I LT/Sv and metaphase II wild-type (wt) oocytes analyzed 15 and 21 h after germinal vesicle breakdown (GVBD). (A) *MI-arrested LT/Sv and MII-arrested wild-type oocytes were analyzed 15 and 21 h after GVBD. The graph shows relative level of mRNA in LT/Sv oocytes in comparison to the mRNA level (100%; dotted line) in wild-type oocytes. The mRNA levels in all samples were standarized against mRNA level of housekeeping gene* (GAPDH). *Error bars depict SEM (standard error of mean). Columns marked with letters are significantly different (P < 0.05).* **(B)** *Representative gel of LT/Sv and wild-type oocytes analyzed 15 h after GVBD. Intensity of bands reflects mRNA level in analyzed groups of oocytes.*

MII wild-type oocytes (261% and 211% of wild-type levels, respectively; P < 0.05; Fig. 5A). However, there was no significant difference in the level of these mRNAs between MI LT/Sv and MII wild-type oocytes analyzed 21 h after GVBD (130% and 109% of wild-type levels; P > 0.05; Fig. 5A). The level of mRNA encoding PKCζ analyzed 15 h after GVBD was lower in metaphase I LT/Sv oocytes than in age-matched metaphase II wild-type oocytes (P < 0.05; Fig. 5A). However, the level of PKCζ mRNA was significantly higher in LT/Sv oocytes collected 21 h in comparison to these collected 15 h after GVBD (85% and 66% respectively; P < 0.05; Fig. 5A) and was similar to that observed in age-matched metaphase II wild-type oocytes (P > 0.05). Our results indicate that the level of PKCα, PKCδ and PKCζ mRNAs differ between LT/Sv and wild-type oocytes collected 15 h after GVBD (Fig. 5 A,B), but the level of these mRNAs becomes similar 21 h after GVBD (Fig. 5A).

Discussion

Ability of LT/Sv oocytes to be activated by sperm develops gradually during prolonged in vitro *culture*

Significant fraction of maturing LT/Sv oocytes arrests at metaphase I stage (Eppig, 1978; Kaufman and Howlett, 1986). Previously we have shown that fertilization of these oocytes does not result in their activation (Ciemerych and Kubiak, 1998). Here we showed that metaphase I arrested LT/SV oocytes gradually (in a time period between 15 and 21 h after GVBD) develop the ability to be activated by sperm. Moreover, LT/Sv oocytes that were induced to complete the first meiotic division and reached metaphase II stage (MII*) acquired the ability to be activated by sperm at the same time point after GVBD as metaphase I-arrested oocytes. Thus, the development of this ability in LT/Sv oocytes depends on cytoplasmic rather than nuclear maturation. These results agree with previous reports on delayed maturation of oocytes of KE strain of mice. KE oocytes are characterized by prolonged metaphase I stage (Polanski, 1986; Polanski, 1995), and complete the first meiotic division a few hours later than oocytes of wild-type mice. Moreover, oocytes that remain arrested at MI for 20 h after GVBD can be activated by sperm similarly to MII-arrested oocytes, which suggests that their cytoplasmic maturation was normal (Polanski, 1995). Oocytes isolated from small antral follicles of sexually immature wild-type [F_1 (C57BL/6J x SJL)] females also become frequently arrested at MI. Such oocytes can be activated by sperm with the same frequency as oocytes that progressed in nuclear maturation and reached metaphase II stage (Eppig *et al.,* 1994). Both MI and MII-arrested wild-type oocytes do not differ in the pattern of protein synthesis, indicating that premature metaphase I arrest does not affect the cytoplasmic maturation (Eppig *et al.,* 1994; McConnell *et al.,* 1995). Our preliminary observations suggest that at least during initial stages of meiotic maturation i.e. until the time when wild-type oocytes become ready to complete the first meiotic division (8-9 h after GVBD), both wild-type and LT/Sv oocytes have similar general pattern of protein synthesis. However, we cannot exclude possibility that the subtle differences in certain proteins level are responsible for different reaction of wild-type and LT/Sv oocytes to fertilization. These differences may involve proteins functioning in the control of calcium release mechanism.

MI-arrested LT/Sv oocytes are defective in the generation of sperm-induced Ca^{2+} oscillations

Fusion of an oocyte and a spermatozoon triggers oscillations of intracellular calcium level (Cuthbertson and Cobbold, 1985). The increase in the intracellular Ca^{2+} is necessary for the activation of several signaling pathways leading to inactivation of CSF and MPF and inducing progression to the embryonic cell cycles (Ducibella *et al.,* 2006). Our study showed that the majority of fertilized MI and MII-arrested LT/Sv oocytes generated only one or two calcium transients in contrast to MII wild-type oocytes that usually generated repetitive Ca^{2+} spikes in response to sperm penetration (Lee *et al.,* 2006). The pattern of Ca^{2+} oscillations observed in fertilized LT/Sv oocytes resembles that which occurred in wild-type oocytes fertilized at the GV stage or during *in vitro* maturation (2-3 h after release from the ovarian follicles). Such oocytes generate fewer Ca^{2+} transients than oocytes fertilized in metaphase II stage (Jones *et al.,* 1995; Cheung *et al.,* 2000). Although the ability to be activated by sperm developed gradually in LT/Sv oocytes during metaphase I arrest, fertilization at 15 or 21 h after GVBD led to the same impaired pattern of Ca^{2+} oscillations. Moreover, even MII LT/Sv oocytes that spontaneously completed the first meiotic division failed to generate long-lasting multiple Ca^{2+} transients. Thus, we confirmed that multiple

calcium transients are not prerequisite for the oocyte activation. As it was previously documented MII-arrested mouse oocytes can be activated by parthenogenetic stimuli such as ethanol that induce only one Ca^{2+} transient (reviewed in Swann and Ozil, 1994). Such parthenogenetic embryos, despite the lack of the Ca^{2+} oscillations, successfully undergo preimplantation and early stages of postimplantation development (Swann and Ozil, 1994; Liu *et al.,* 2002; Rogers *et al.,* 2006). However, abnormally low number of Ca^{2+} transients observed by us in LT/Sv oocytes suggests that the signaling pathways operating at the time of fertilization may be anomalous in these oocytes and hinder their sperm-induced activation. It is known that these pathways involve the release of Ca^{2+} from endoplasmic reticulum. This process is mediated by inositol 1,4,5 – triphosphate (IP_3) - a product of enzymatic cleavage of phosphatidylinositol 4,5 – biphosphate (PIP_2) by sperm-specific phospholipase ζ (PLCζ) (Saunders *et al.,* 2002; Swann *et al.,* 2004). Pattern of Ca^{2+} oscillations generated after fertilization depends on the increase in the amount of type I IP_3 receptors (IP_3R-1) during oocyte maturation (Mehlmann *et al.,* 1996; Parrington *et al.,* 1998; Fissore *et al.,* 1999; Xu *et al.,* 2003). Xu *et al.* (2003) showed that the inhibition of IP_3R-1 accumulation in wild type oocytes led to the decrease in the number of Ca^{2+} transients. We showed the same trend for LT/Sv oocytes. However, in our study we did not detect any significant differences in the level of mRNA encoding IP_3R-1 between MII wild-type and age-matched MI LT/Sv oocytes, which suggests that in oocytes of LT/Sv mice this part of the calcium-dependent signal transduction pathway remains normal.

Development of the ability of LT/Sv oocytes to be activated by sperm might depend on function of PKCs

Functional meiotic apparatus is crucial for the oocyte activation after fertilization (Winston *et al.,* 1995). The stability of the meiotic spindle in maturing mouse oocytes is regulated by PKCζ, the only PKC isoform localized at the microtubules of metaphase II spindle (Page Baluch *et al.,* 2004). Inhibition of PKCζ activity leads to misalignment of chromosomes and disruption of metaphase II spindle (Page Baluch *et al.,* 2004). The role of PKCζ in the regulation of metaphase I spindle assembly has not been examined yet, so it cannot be excluded that this isoform might be responsible for the integrity of metaphase I spindle not only in wild-type but also in LT/Sv oocytes. We showed that the level of PKCζ mRNA is lower in MI-arrested LT/Sv oocytes analyzed 15 h after GVBD as compared with age-matched MII wild-type oocytes. Several reports suggest that PKCζ can influence spindle stability by affecting the MAP kinase pathway (Schonwasser *et al.,* 1998) that participates in the establishment of the M-phase array of microtubules during meiotic maturation (Verlhac *et al.,* 1996; Lefebvre *et al.,* 2002; Terret *et al.,* 2003). It has been reported previously that the MI spindle is unstable in aging LT/Sv oocytes (Albertini and Eppig, 1995; Ciemerych and Kubiak, 1998). Thus, it is possible that the decrease in the level of PKCζ observed in LT/Sv oocytes 15 h after GVBD may influence function of their metaphase I spindle and consequently restrict their ability for sperm-induced activation.

Another isoform of PKC – PKCδ - was also found to be enriched at the metaphase II spindle in mouse oocytes, however, in contrast to PKCζ, PKCδ seems to be tightly bound not to the microtubules but to the chromosomes (Viveiros *et al.,* 2001; Viveiros *et al.,* 2003; Page Baluch *et al.,* 2004). PKCδ become associated with microtubules during the first meiotic division (Viveiros *et al.,* 2001; Viveiros *et al.,* 2003). Localization of PKCδ to metaphase I spindle in both wild-type and LTXBO oocytes (which have similar defects like LT/Sv oocytes), prompted Viveiros and co-workers to hypothesize that PKCδ is a major PKC isoform involved in regulation of MI/MII transition which functions by delaying the entry of oocytes into anaphase I (Viveiros *et al.,* 2001; Viveiros *et al.,* 2004). Consistent with this hypothesis, treatment of MI-arrested LTXBO oocytes with the PKCs inhibitor reduced MPF activity and promoted their progression to metaphase II stage (Viveiros *et al.,* 2001). Our studies show that the level of PKCδ mRNA was 2.5 times higher in LT/Sv oocytes than in wild-type oocytes analyzed 15 h after GVBD. Twenty-one hours after GVBD the levels of PKCδ mRNA in LT/Sv and wild-type oocytes were similar suggesting that this class of mRNA underwent extensive deadenylation or/and degradation during the final stages of meiotic maturation. This observation seems to be consistent with previous findings that PKC activity was the highest in oocytes collected about 14 h after GVBD and then decreased (Viveiros *et al.,* 2001). The abnormally high level of PKCδ mRNA found in LT/Sv oocytes arrested at metaphase I suggests that this isoform of PKC may indeed be involved in delaying the anaphase onset in LT/Sv oocytes and therefore limit their ability to be successfully activated by sperm.

We found elevated level of PKCα mRNA in LT/Sv oocytes analyzed 15 h but not 21 h after GVBD when compared to PKCα mRNA level in MII wild-type oocytes. PKCα was proposed to sustain long-lasting Ca^{2+} oscillations in fertilized oocytes via the regulation of store-operated Ca^{2+}-influx (Halet, 2004). However, in our study we did not notice any direct correlation between the level of PKCα mRNA and the number of oscillations generated in oocytes after fertilization. The number of oscillations was always higher in wild-type oocytes characterized by relatively lower level of PKCα mRNA. Since PKCα belongs to subfamily of PKC sensitive to Ca^{2+}, our observation supports the hypothesis of Tatone and co-workers that Ca^{2+}-independent rather than Ca^{2+}-dependent PKCs are activated in fertilized oocytes (Tatone *et al.,* 2003).

As mentioned above, different PKCs are believed to be involved in regulation of both meiotic maturation and response to fertilization (Halet, 2004). Our results showed significant differences in the levels of mRNAs encoding three of PKC isoforms, PKCα, PKCδ and PKCζ between LT/Sv and wild-type oocytes analyzed 15 h but not 21 h after GVBD. Disparities in level of PKCs mRNAs detected in MI LT/Sv and MII wild-type oocytes 15 h after GVBD oocytes confirm that PKC in LT/Sv oocytes may be deregulated. Perhaps this deregulation is responsible for delaying the MI/MII transition in these oocytes (Viveiros *et al.,* 2001). Since level of polyadenylated mRNA reflects its translational activity (Cui and Kim, 2007), we can assume that also pattern of synthesized proteins may differ in LT/Sv and wild-type oocytes. Differences in mRNAs levels between both groups of oocytes disappear within 21 h after GVBD. We also showed that 21 h after GVBD MPF activity is slightly lower in MI-arrested LT/Sv oocytes in comparison to the oocytes 15 h after GVBD and this correlates with the development of the ability for sperm-induced activation in LT/Sv oocytes. However, we believe that the age-associated decrease in MPF activity is not crucial for activation of LT/Sv oocytes since majority of such metaphase I-arrested oocytes can be activated by parthenogenetic agents as early as 10 h after GVBD despite the presence of stable MPF

(Ciemerych and Kubiak, 1998). This observation suggests again that the signaling cascades operating during fertilization differ in LT/Sv oocytes analyzed 15 and 21 h after GVBD.

Genetic diversity of LT/Sv strain mice

Our results showed that in addition to already described abnormalities the LT/Sv oocytes are also characterized by the defective calcium oscillations and delayed processes of cytoplasmic maturation. However, the molecular basis of these phenomena remains unknown. Several research groups investigated genetic linkage between different genes and meiotic abnormalities observed in LT/Sv oocytes trying to uncover mutations responsible for this complex phenotype. West and co-workers believe that the culprit is the co-dominant autosomal gene *'poo'* (primary oocyte ovulation; West *et al.,* 1993). However, the results of other studies indicate that the phenotype of LT/Sv oocytes depends on the function of multiple genes (Eppig *et al.,* 1996; Lee *et al.,* 1997; Everett *et al.,* 2004). It is possible that the incomplete penetration and variable expression of these genes lead to a great genetic heterogeneity of LT/Sv mice and make genetic mapping and understanding of LT/Sv phenotype difficult (West *et al.,* 1993; Lee *et al.,* 1997; Everett *et al.,* 2004). Similarly, at present there is no indication what genes or mutations are responsible for the LT/Sv phenotype described by us in this paper.

Materials and Methods

Chemicals

Unless otherwise stated, reagents were obtained from Sigma-Aldrich Chemical Company (Poland).

Collection and culture of oocytes at germinal vesicle stage

Two- to three-month-old LT/Sv or wild-type, i.e. F_1 (C57Bl/10 x CBA/H) female mice were injected intraperitoneally with 10 IU pregnant mare serum gonadotrophin (PMSG; Folligon, Intervet, Netherlands) to stimulate the development of ovarian follicles. Forty-eight to fifty-two hours later females were killed by cervical dislocation. Fully-grown oocytes arrested at prophase of the first meiotic division (germinal vesicle stage; GV) were released from ovarian follicles. Oocytes were freed from cumulus cells by pipetting and then cultured for 2 h in M2 medium (medium M16 buffered with HEPES; Fulton and Whittingham, 1978) containing bovine serum albumin (BSA; 4 mg/ml). Only oocytes that underwent germinal vesicle breakdown (GVBD) within first 2 h of *in vitro* culture were used for further experiments.

Experimental induction of the first meiotic division in metaphase I LT/Sv oocytes

LT/Sv oocytes that failed to complete first meiotic division within 10 h after GVBD were placed for 5 min in solution of Ca^{2+} ionophore A23187 (5 μM solution in M2 medium) and subsequently were incubated for 30 min in M2 medium containing 5 mM of unspecific kinase inhibitor 6-dimetyhyloaminopurine (6-DMAP) (Hirao and Eppig, 1999). Subsequently oocytes were washed in M2 medium. Oocytes that completed the first meiotic division and reached metaphase II stage (hereinafter called metaphase II*, MII*) within 90 min following ionophore and the 6-DMAP treatment were cultured in M2 medium before further experiments.

In vitro *fertilization of oocytes*

Spermatozoa from caudae epididymides of mature F_1 (C57Bl/10 x CBA/H) males were suspended in 0.5 ml of fertilization medium (Fraser and Drury, 1975) containing 4 mg/ml of BSA (Pentex, Miles, USA). Sperm concentration was approximately $2x10^7$ spermatozoa/ml. Spermatozoa were incubated for 2 h at 37.5ºC under 5% CO_2 in air to allow capacitation and spontaneous acrosome reaction. After incubation the sperm suspension was transferred to a tube, centrifuged (5 min, 1700 x g) and then placed back in incubator for 10 min to enable sperm dispersion. Spermatozoa from the upper layer of the supernatant were regarded as the most vigorous and only these were used for oocyte insemination.

LT/Sv (MI, MII*) and F_1 (MII) oocytes were fertilized at 15 or 21 h after GVBD. Prior to fertilization zonae pellucidae were removed by brief (<1 min) exposure to acidic Tyrode's solution (pH 2.5; Nicolson *et al.,* 1975). Zonae-free oocytes were washed in M2 medium, transferred to 100 μl droplet of fertilization medium and then 1 μl of sperm suspension was added. Insemination was carried out at 37.5ºC under 5% CO_2 in air for 30 min. Subsequently oocytes were gently pipetted to remove loosely attached spermatozoa, cultured for 6 h in DMEM medium (Invitrogen, USA) supplemented with 10% of fetal bovine serum (Invitrogen, USA), 50 units/ml of penicillin and 50 μg/ml of streptomycin, and fixed for cytological examination. Oocyte activation was assessed by the presence of the first (for MI oocytes) or second (for MII* oocytes) polar bodies and pronuclei. Each of the experimental variants was repeated three times.

Measurement of intracellular Ca^{2+}

To monitor changes in the level of intracellular Ca^{2+}, LT/Sv (MI, MII) and F_1 (MII) oocytes were incubated in 2 μM solution of Ca^{2+}-sensitive fluorescent probe, fura-2 AM (Molecular Probes, Netherlands) in M2 medium for 30 min at 37.5°C under 5% CO_2 in air (Baltz and Phillips, 1999). After loading with fura-2 AM, oocytes were washed in M2, released from zonae pellucidae by an exposure to acidic Tyrode's solution and transferred to heated (37°C) chamber (Chance Proper LTD, Smethwick, UK) containing M2 without BSA installed on an inverted microscope stage (Diaphot, Nikon, Japan). One microliter of capacitated sperm suspension was added in a close proximity of oocytes. Sperm penetration was judged by the rise of intracellular Ca^{2+} within the oocyte cytoplasm. Measurements were made during 90 min after sperm penetration into the oocyte. Two excitation wavelengths of 340 nm and 380 nm were used sequentially every 10 s with the help of an excitation filter wheel (Lep Ludl, USA), and emission was recorded at 510 nm (interference filters as well as dichroic mirror from Chroma Inc., USA). Images were acquired using 10x objective and PCC (Photon Counting Camera, Retiga 1300, Q Imaging, Canada) and ratiometrically processed using AQM Advance v. 6.0 software (Kinetic Inc, UK). The experiment was repeated three times.

Histone H1 kinase assay

Histone H1 kinase activity (the biochemical indicator of MPF activity) was determined in *in vitro* cultured MII F_1 and MI LT/Sv oocytes 15 and 21 h after GVBD. Groups of 5 oocytes were transferred in 1 μl of PBS into microcentrifuge tubes, immediately freezed and stored at -80ºC. Next, 3 μl of lysis buffer [0.16 M glicerophosphate, 40 mM EGTA (pH 7.3), 30 mM $MgCl_2$, 2 mM DTT, Complete Protease Inhibitor Cocktail (Roche, Germany), and BSA (11 mg/ml)] were added to each sample. Samples were lysed by 3 rounds of freezing and thawing. Next, 1.5 μl of reaction buffer (0.5 mg/ml histone H1, 5 mM ATP and 1.67 μCi/μl [^{32}P]-ATP (ICN, Prospecta, Poland) was added to each sample. Samples were incubated for 30 min in 30ºC. The reaction was stopped by addition of Laemmli buffer (Laemmli, 1970) Samples were boiled for 10 min and separated in12% SDS-PAGE. Gels were exposed to autoradiography films (BioMax MS Film, Kodak) at -80ºC for 24-72 h. Intensity of autoradiography bands was measured with GelDoc apparatus and Quantity One 4.2.2 software (Biorad, Hercules, Canada). Intensity of bands reflected the activity of histone H1 kinase i.e. the relative amount of ^{32}P incorporated into exogenous histone H1. The experiment was repeated four times.

RT-PCR

Groups containing 25 of MII F_1 and MI LT/Sv oocytes were collected 15 and 21 h after GVBD and placed in 20 μl of lysis buffer (DYNAL, Oslo, Norway). Samples were frozen and stored at -80ºC. mRNA was isolated from oocytes using the Dynabeads mRNA DIRECT Micro Kit (DYNAL,

Oslo, Norway) according to the manufacturer's protocol. For single sample 20 μl of paramagnetic oligo-$(dT)_{25}$ beads suspension was used. To bind mRNA to the oligo-$(dT)_{25}$ beads the mixture was rotated for 45 min, pelleted and then supernatant was discarded. The reverse transcription was performed in 20 μl of reaction mixture containing 200 U of Superscript II Reverse Transcriptase (Invitrogen, USA). Resulting cDNA was then divided into 5 samples (containing 4 μl of cDNA) and PCR amplified (thermocycler PTC-200; MJ Research, Watertown, MA, USA) in the presence of forward and reverse primers specific for following coding sequences: *IP_3R-1* (Xu *et al.,* 2003), *PKCα, PKCδ and PKCζ* (Freire-Moar *et al.,* 1991). The levels of the PCR products were compared to the level of the PCR product of control housekeeping gene - *GAPDH* (Wrobel *et al.,* 2007). The number of cycles was experimentally chosen as the lowest number needed to obtain the product that could be visualized in agarose gel. Reactions were performed under following conditions: 94ºC for 1 min, 59ºC for 1 min, 72ºC for 1 min; 45 cycles (PKCα, PKCδ and PKCζ) or 35 cycles (GAPDH and IP_3R-1); the last cycle was followed by a 10 min extension at 72ºC. The products of PCR were separated by electrophoresis in 1.5 % agarose gel (Roche, Germany). RT-PCR method was considered to be the semi-quantitative. Values obtained for mRNA encoding PKC isoforms and IP3R were then compared to the values of GAPDH that were denoted as 100%. Next, the mean ratio between values obtained for LT/Sv and wild-type oocytes was presented as a proportion of 100%. Intensity of bands reflecting level of mRNA present in oocytes was measured densitometrically with GelDoc using software Quantity One 4.2.2 (Biorad, Hercules, Canada). Each RT-PCR analysis was repeated three times.

Cytological examination

LT/Sv (MI, MII*, MII) and F_1 (MII) oocytes were fixed with Heidenhein's fixative 6 h after fertilization. Whole-mount preparations were prepared and then stained with hematoxylin according to the method of Tarkowski and Wroblewska (1967).

Photographic documentation and statistical analysis

Oocytes were analyzed and photographed using Zeiss Axiovert inverted microscope equipped with a digital camera (Pixelfly, PCO). Statistical analysis of the results was performed using χ^2 test, Fisher's exact test and t-Student test.

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