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Mitogen-activated protein kinase regulates normal transition from metaphase to interphase following parthenogenetic activation in porcine oocytes

Hideki Tatemoto and Norio Muto
School of Bioresources, Hiroshima Prefectural University, Shobara, Hiroshima 727-0023, Japan

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Summary

The decrease in maturation-promoting factor (MPF) activity precedes that in mitogen-activated protein kinase (MAPK) activity after egg activation, but the cellular functions of this delayed inactivation of MAPK are still unclear. The present study was conducted to examine the essential role of MAPK activity for supporting the transition from metaphase to interphase in porcine oocytes matured *in vitro*. The increases in the phosphorylated forms of MAPK and the activities of MAPK and histone H1 kinase (H1K) were shown in oocytes arrested at the metaphase II (MII) stage. After additional incubation of MII-arrested oocytes in medium with added U0126, a specific inhibitor of MAPK kinase, 24% of oocytes completed the second meiotic division and underwent entry into interphase with pronucleus (PN) formation, but not second polar body (PB-2) emission. The intensities of the phosphorylated forms of MAPK and the activities of MAPK and H1K in matured oocytes treated with U0126 were significantly decreased by the treatment with U0126. Electrostimulation to induce artificial activation caused both H1K and MAPK inactivation; the inactivation of H1K preceded the inactivation of MAPK and sustained high levels of MAPK activity were detected during the period of PB-2 emission. However, the time sequence required for MAPK inactivation was significantly reduced by the addition of U0126 to the culture medium following electrostimulation, resulting in the dramatic inactivation of MAPK distinct from that of H1K. In these oocytes, PB-2 emission was markedly inhibited but little difference was found in the time course of PN formation compared with oocytes not treated with U0126. These findings suggest that the decrease in MAPK activity is partly involved in driving matured oocytes out of metaphase to induce PN development, and that the delayed MAPK inactivation after the onset of MPF inactivation in activated oocytes has a crucial role for PB-2 emission to accomplish the transition from meiosis to mitosis.

Key words: Egg activation, MAP kinase, Pig oocyte, Pronuclear formation, Second polar body emission

Introduction

Mammalian oocytes arrest at metaphase II (MII) following resumption of meiosis. These oocytes possess elevated levels of maturation- (or M-phase-) promoting factor (MPF) activity and mitogen-activated protein kinase (MAPK) activity. MPF has been identified as a heterodimeric protein kinase composed of a catalytic subunit $p34^{cdc2}$ kinase, which is the homologue of the yeast cdc2/CDC28 protein kinase, and a regulatory subunit cyclin B (Nurse & Thuriaux, 1980; Dunphy *et al*., 1988; Gautier *et al*., 1988). The activation of $p34^{cdc2}$ kinase induces entry into M-phase in multicellular eukaryotes (Dunphy *et al*., 1988; Gautier *et al*., 1988). This protein kinase activity, referred to as histone H1 kinase (H1K) activity, increases as cells enter M-phase (Gautier *et al*., 1989, 1990). Cytostatic factor (CSF) is postulated to maintain the elevated levels of MPF activity (Masui & Markert, 1971; Masui, 1991) and hence may be responsible for meiotic arrest at the MII stage (Sagata *et al*., 1989). MAPK, which is also termed extracellular-regulated kinase (ERK), may be involved in CSF activity (Haccard *et al*., 1993). MAPK is a serine/threonine kinase of 42 and 44 kDa, and acti-

All correspondence to: Hideki Tatemoto, School of Bioresources, Hiroshima Prefectural University, Shobara, Hiroshima 727-0023, Japan. Tel: +81 8247 4 1746. Fax: +81 8247 4 0191. e-mail: hidettmt@bio.hiroshima-pu.ac.jp

vated by phosphorylation on both tyrosine and serine/threonine residues by an upstream kinase, MAPK/ERK kinase (MAPKK, MEK), which in turn is phosphorylated by Raf or a c-*mos* proto-oncogene product (Mos) (Fabian *et al*., 1993; Gotoh & Nishida, 1995; Kosako *et al*., 1996). In somatic cells, the MAPK cascade plays a critical role in diverse intracellular signalling processes of the cell cycle triggered by external signals (Nishida & Gotoh, 1993), and MAPK appears to be specifically activated during meiosis but not during G_2/M transition in mitosis.

MPF in MII-arrested oocytes is inactivated after parthenogenetic activation or fertilisation, (Collas *et al*., 1993; Liu *et al*., 1998; Liu & Yang, 1999). The inactivation of MAPK has also been shown to be involved in the regulation of pronuclear envelope assembly/disassembly after fertilisation in mouse oocytes (Moos *et al*., 1995, 1996). Following either egg activation or fertilisation in mouse (Verlhac *et al*., 1994; Moos *et al*., 1995) and bovine (Liu *et al*., 1998, Liu & Yang, 1999) oocytes, the decline in H1K activity precedes that in MAPK activity. Yamauchi *et al*. (1998) also observed that the decrease in H1K activity precedes that in MAPK activity after egg activation in porcine oocytes. According to Liu *et al*. (1998), the decrease in H1K activity is involved in the initiation of egg activation, i.e. the exit from MII, whereas the decrease in MAPK activity correlates with onset of pronucleus (PN) formation, showing that inactivation of MPF and MAPK probably occurs via two independent processes. However, the role of MAPK in the first embryonic cell cycle is not well understood. In particular, little is known of the importance of the delayed inactivation of MAPK compared with MPF inactivation after egg activation in supporting the metaphase-to-interphase transition.

To determine whether the MAPK cascade is involved in egg activation in MII-arrested porcine oocytes, oocytes matured *in vitro* were further treated in medium to which had been added a specific inhibitor of MEK, U0126 (Duncia *et al*., 1998; Favata *et al*., 1998). We also examined the time course of changes in MAPK and MPF activities as well as nuclear status during parthenogenetic activation in porcine oocytes with or without U0126 treatment following electrostimulation to clarify the critical role of delayed MAPK inactivation after the onset of MPF inactivation in the transition from metaphase to interphase.

Materials and methods

All chemicals were purchased from Sigma Chemical Company (St Louis, MO) unless otherwise stated.

Collection of oocytes

Ovaries were collected from maturing gilts at a local slaughterhouse and transported to the laboratory in 0.9% (w/v) NaCl containing 100 mg/l kanamycin sulphate (Meiji Seika, Tokyo, Japan) at 30 °C. Within 2 h of slaughter the follicular contents were recovered by excising the visible small antral follicles (about 2–5 mm in diameter) on the ovarian surface using a razor, and by scraping the inner surface of the follicle walls with a disposable surgical blade. Only cumulus–oocyte complexes (COCs) with uniform ooplasm and a compact cumulus cell mass were collected and washed three times with HEPES-buffered Tyrode's medium containing 0.01% (w/v) polyvinyl alcohol (H-TL-PVA; Funahashi *et al*., 1994).

Maturation culture of oocytes

The basal medium for maturation culture of oocytes was BSA-free NCSU37 (NCSU37; Petters & Wells, 1993) supplemented with 0.6 mM cysteine, 2% (v/v) MEM amino acids solution (Gibco BRL, Grand Island, NY), 1% (v/v) MEM non-essential amino acids solution (Gibco), 0.04 units/ml oFSH, 0.02 units/ml oLH and 10% (v/v) porcine follicular fluid. After washing in basal medium, groups of 20 COCs were transferred into 100 µl droplets of the basal medium, which had previously been equilibrated in a CO_2 incubator. After 20 h of maturation culture, the oocytes were washed and transferred to 100 µl droplets of the basal medium without hormonal supplementation for an additional 24 h of culture. All media containing COCs were covered with mineral oil and cultured at 39 °C in an atmosphere of 5% CO_2 in air. After total maturation culture of 44 h, COCs were sucked through a narrow-bore pipette to remove their cumulus cells in H-TL-PVA containing 0.1% (w/v) hyaluronidase, and the denuded oocytes with a polar body were selected for the subsequent experiment.

Electrostimulation of oocytes

Current pulses for stimulation of oocytes were provided using a cell fusion apparatus (SSH-1; Shimadzu, Kyoto, Japan). The oocytes matured *in vitro* were transferred to electroporation medium (Tatemoto & Terada, 1999) and stimulated twice by DC pulses of 60 V/mm for 30 µs with a parallel stainless steel wire chamber spaced 1 mm apart on a glass slide at 2 min intervals. Electrostimulated oocytes were further cultured in NCSU37 medium as described below.

Evaluation of nuclear status

At the end of culture, the oocytes were mounted, fixed

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in acetic acid–ethanol (1:3, v/v) for 72 h, stained with 1% (w/v) lacmoid in 45% (v/v) acetic acid, and examined for determination of their nuclear status using a phase-contrast microscope at ×400 magnification. Oocytes that had progressed beyond anaphase II were taken to be activated.

H1K and MAPK assay

H1K and MAPK activities of the oocytes were determined by the method of Inoue *et al*. (1996). Briefly, 10 denuded oocytes were put into microtubes containing 10 µl extraction buffer composed of 15 mM EGTA, 1% Nonidet P-40, 60 mM sodium β-glycerol phosphate, 30 mM *p*-nitrophenyl phosphate, 25 mM MOPS (pH 7.2), 15 mM $MgCl_2$, 0.2 mM Na_3VO_4, 1 mM dithiothreitol (DTT), 2 µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml pepstatin, 1 mM phenylmethylsulphonylfluoride and 50 µM *p*-aminobenzoic acid, and frozen at –80 °C until use. The lysate (10 µl) was added to 5 µl of 2.5 µM cAMP-dependent protein kinase inhibitor (TTYADFIASGRTGRRNAIHD), 5 µl of 0.1 mM [γ-^{32}P]ATP (14.8 MBq/ml; Amersham, Arlington Heights, IL) and 5 µl of 2 mg/ml myelin basic protein (MBP) as a substrate for MAPK, or 5 mg/ml histone H1 for H1K. Kinase reactions commenced with the addition of [γ-^{32}P]ATP and performed at 30 °C for 40 min (for MAPK) or at 37 °C for 20 min (for H1K). Assays were terminated with 0.4 ml of 20% (w/v) trichloroacetic acid solution (TCA) and 0.1 ml of 1% (w/v) bovine serum albumin as a carrier protein for precipitation. After centrifugation at 12 000 *g* for 5 min, the precipitates were washed twice with 0.4 ml of 20% TCA and dissolved in 0.2 ml of 1 M NaOH. Radioactivity was counted using a liquid scintillation counter (LSC-1000, Aloka, Tokyo, Japan). Kinase activity was determined by subtracting the value of blank tubes containing all materials except for lysed oocytes from each value.

Immunoblotting analysis of MAPK (ERK1/2)

Phosphorylation status of ERK1/2 was detected by immunoblotting with polyclonal anti-phospho-specific p44/42 MAPK (Thr202/Tyr204) antibody (New England Biolabs, Beverly, MA). This was carried out in advance to confirm the cross-reactivity of this antibody with porcine phosphorylated (active) forms of ERK1/2, which were detected using anti-MAPK polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). This pre-test confirmed that the mobility-shifted ERK1/2 bands, resulting from phosphorylation, were selectively recognised by anti-phospho-specific p44/42 MAPK antibody (Fig. 1). Twenty oocytes were directly put into a microtube containing 4 µl of SDS sample buffer (pH 7.8) composed of 10 mM Tris-HCl, 15% (w/v) sucrose, 5% (v/v) β-mercaptoethanol,

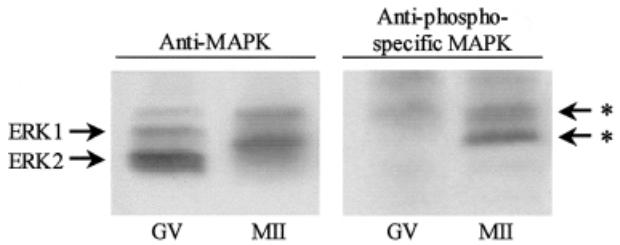

Figure 1 Detection of ERK1 and ERK2 (arrows) by immunoblotting. A total of 20 oocytes was loaded into each lane. Porcine oocytes at the germinal vesicle (GV) stage and the metaphase II (MII) stage were collected, and subjected to immunoblotting with anti-MAPK or anti-phospho-specific p44/42 MAPK polyclonal antibody. GV oocytes and MII oocytes were cultured for 0 h and 44 h, respectively. The electrophoretically retarded bands represent the phosphorylated (activated) forms of ERK1/2 (arrows with an asterisk). By incubation with anti-MAPK antibody, the mobility-shifted ERK1/2 bands were detected following oocyte maturation, but only phosphorylated bands of ERK1/2 were selectively recognised by anti-phospho-specific p44/42 MAPK antibody. The results are representative of four independent experiments.

2.5% (w/v) SDS and 1 mM EDTA. After denaturation at 100 °C for 3 min, the proteins were separated by SDS-PAGE on a 12.5% polyacrylamide gel (PhastGel, Pharmacia LKB, Uppsala, Sweden) using the PhastSystem (Pharmacia), and then transferred to nitrocellulose membranes (Hybond ECL Western; Amersham) using the PhastTransfer (Pharmacia). Following electrophoretic transfer, the phosphorylated ERK1/2 bands were detected with the anti-phospho-specific p44/42 MAPK antibody in an ECL Western blotting analysis system (Amersham) according to the manufacturer's instructions.

Experimental design

Experiment 1 was carried out to determine the possible contribution of the decrease in MAPK activity to egg activation in matured oocytes. After 44 h of *in vitro* maturation in basal medium, the mechanically denuded oocytes were further incubated for 20 h in basal medium with or without 10 µM U0126 (Promega, Madison, WI). A stock solution of U0126 was prepared in dimethyl sulphoxide (DMSO) and the solvent was added to every culture at a final concentration of 0.1%. At the end of culture, the oocytes were examined for their nuclear status, the phosphorylated forms of ERK1/2 on SDS-PAGE and the activities of MAPK and H1K.

Experiment 2 was conducted to examine whether MAPK activity after egg activation has an influence on the transition from metaphase to interphase. Oocytes stimulated by electrical pulses were cultured in

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NCSU37 with or without 10 μM U0126 for 10 h. Time course changes in nuclear status, the phosphorylated forms of ERK1/2, and the activities of MAPK and H1K were analysed at 0, 1, 2, 4, 6, 8 and 10 h after electrostimulation.

Statistical analysis

Statistical analyses of data from four replicate trials for each treatment comparison were carried out by ANOVA and Fisher's protected least significant difference test using the STATVIEW program (Abacus Concepts, Berkeley, CA). All percentage values were subjected to arcsine transformation before statistical analysis. Values are expressed as mean ± SEM. A probability of $p < 0.05$ was considered to be statistically significant.

Results

Effects of U0126 on meiotic arrest in matured oocytes

Treatment of matured oocytes with the MEK inhibitor, U0126, induced egg activation followed by PN formation, but not second polar body (PB-2) emission. At 20 h after the addition of U0126 to medium, 24.3% of oocytes ($n = 107$) were released from meiotic arrest and developed into single PN. In contrast, the activation rate of oocytes cultured without U0126 (aged oocytes) was extremely low (1.7%, $n = 121$). The intensities of phosphorylated (activated) bands of ERK1/2 in oocytes treated with U0126 were obviously decreased compared with those of matured and aged oocytes (Fig. 2). The activities of MAPK and H1K in matured oocytes were 10.2 ± 0.4 and 7.9 ± 0.4 fmol/min per oocyte, respectively, and these kinase activities did not

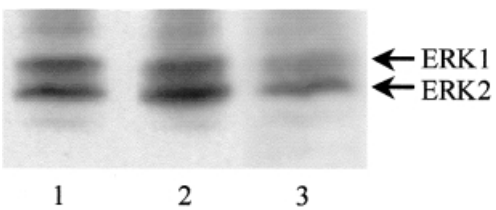

Figure 2 Changes in phosphorylated forms of ERK1/2 during additional incubation of matured oocytes for 20 h in the absence or presence of 10 μM U0126. A total of 20 oocytes was loaded into each lane. Blots were incubated with anti-phospho-specific p44/42 MAPK antibody. Lane 1, oocytes cultured for 44 h (matured oocytes); lane 2, oocytes cultured for 44 h and then cultured without U0126 for an additional 20 h (aged oocytes); lane 3, oocytes cultured for 44 h and then treated with U0126 for an additional 20 h (treated oocytes). The finding is representative of four independent experiments.

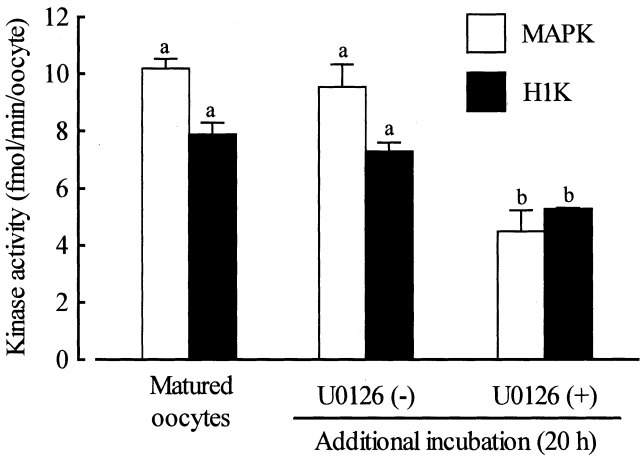

Figure 3 Changes in the activities of MAPK and H1K during additional incubation of matured oocytes for 20 h in the absence (–) or presence (+) of 10 μM U0126. The values are expressed as mean ± SEM. [a,b]Within the same category, values with different superscripts are significantly different ($p < 0.05$).

change throughout the 20 h additional incubation without U0126 (Fig. 3). However, the treatment with U0126 caused a significant decline in MAPK activity (4.5 ± 0.7 fmol/min per oocyte) with a concomitant decrease in H1K activity (5.3 ± 0.1 fmol/min per oocyte) ($p < 0.05$). These findings indicate that MEK inhibitor can drive matured oocytes out of metaphase to induce PN development in combination with MPF and MAPK inactivation.

Effects of U0126 on the transition from metaphase to interphase induced by electrostimulation

To determine whether inactivation of MAPK and MPF was correlated with the morphological changes in porcine oocytes during metaphase-to-interphase transition, the time course of changes in nuclear status, the phosphorylated forms of ERK1/2, and the activities of MAPK and H1K were examined in the oocytes with or without U0126 treatment following electrostimulation. The addition of U0126 caused no difference in the incidence of egg activation at 10 h after electrostimulation (Table 1). Although PB-2 emission was shown in 68.2% of untreated oocytes (control), this rate was significantly lower in oocytes treated with U0126 (13.2%) ($p < 0.05$). In contrast, the incidence of 2PN formation was significantly increased by U0126 treatment (30.8%) compared with controls (11.2%) ($p < 0.05$). In the absence of U0126, PB-2 emission was found in 28.0% of oocytes at 1 h after electrostimulation and this rate was significantly increased to 52.3% after 2 h ($p < 0.05$) (Fig. 4*A*). PN formation (1PN + 2PNs) was first detected at 4 h after electrostimulation (11.4%) and the proportion

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Table 1 Effects of U0126 on the morphological changes in electrostimulated oocytes

U0126 (μM)	No. of oocytes	Percentages of oocytes (mean ± SEM)				
		Activated oocytes	PB-2 emission	PN formation		
				Total	1PN	2PNs
0 (control)	132	85.5 ± 1.2	68.2 ± 2.3	75.1 ± 1.4	63.9 ± 3.9	11.2 ± 3.2
10	141	88.4 ± 1.1	13.2 ± 3.3*	88.4 ± 1.1*	57.5 ± 3.7	30.8 ± 4.4*

PB-2, second polar body; 1PN, single pronucleus; 2PNs, two pronuclei.
* Significantly different from control ($p < 0.05$).

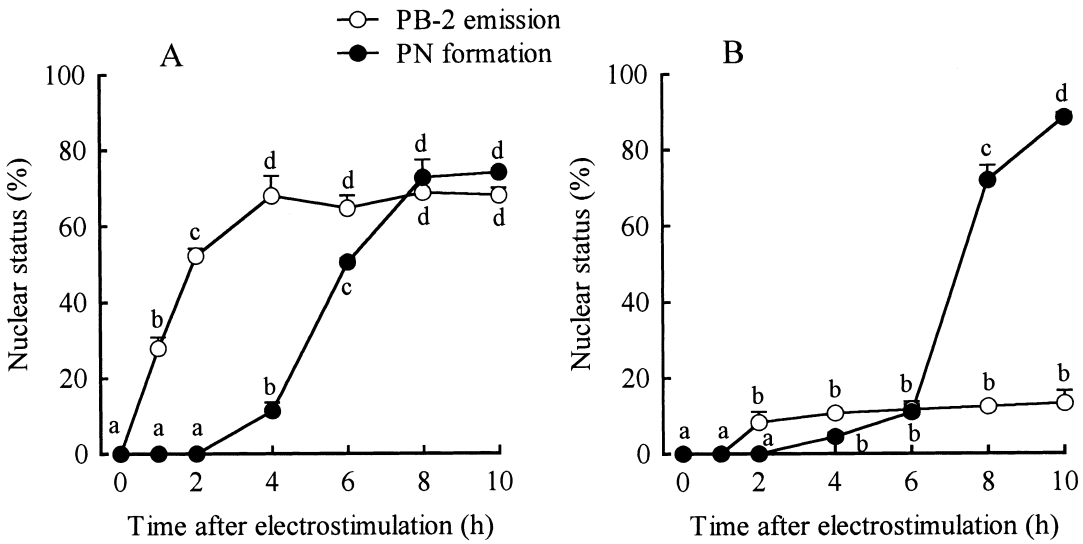

Figure 4 Time course changes in second polar body (PB-2) emission and pronuclear (PN) formation of porcine oocytes treated without (*A*) or with (*B*) 10 μM U0126 following electrostimulation. The findings are expressed as mean ± SEM. Total number of oocytes examined was about 130 at each time point for each experimental group. [a–d]Within the same category, values with different superscripts are signifcantly different ($p < 0.05$).

of PN formation progressively increased and approached a maximum at 8 h after electrostimulation (72.7%). However, in oocytes treated with U0126, PB-2 emission was not observed until 2 h after electrostimulation (8.3%) regardless of the progression beyond anaphase II in 35% of oocytes, and the rate of PB-2 emission showed no increase until 10 h after electrostimulation (Fig. 4*B*). PN formation was a little delayed by U0126 treatment, and culture periods of greater than 8 h after electrostimulation were required to complete PN formation in 71.9% of oocytes treated with U0126.

The quantities of phosphorylated forms of ERK1/2 progressively decreased with prolonged incubation time after electrostimulation (Fig. 5). At 6 h after electrostimulation the intensities of phosphorylated ERK1/2 bands in the oocytes treated with U0126 were slightly reduced compared with controls. Although other distinct differences were not detected in the phosphorylated forms of ERK1/2 on SDS-PAGE between treatments with and without U0126, significant differences were confirmed in the time-dependent decrease in MAPK activity following electrostimulation. MAPK activity in untreated oocytes was sustained at higher levels until 1 h after electrostimulation (10.0 ± 0.1 fmol/min per oocyte) and gradually declined from 2 to 8 h (Fig. 6*A*). However, the addition of U0126 following electrostimulation caused a marked reduction in MAPK activity (Fig. 6*B*). At 1 h after electrostimulation MAPK activity had declined significantly to 3.8 ± 0.1 fmol/min per oocyte as a result of treatment with U0126. MAPK activities both in oocytes treated with U0126 and in untreated oocytes reached minimum levels with the advance of completing PN formation. However, there was no difference in the decreasing patterns of H1K activities between the oocytes with or without U0126 treatment following electrostimulation. A significant decline in H1K

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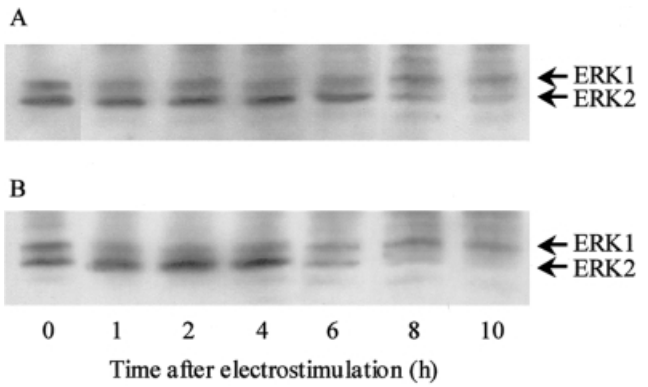

Figure 5 Time course changes in phosphorylated forms of ERK1/2 in porcine oocytes treated without (*A*) or with (*B*) 10 μM U0126 following electrostimulation. A total of 20 oocytes was loaded into each lane. Blots were incubated with anti-phospho-specific p44/42 MAPK antibody. The findings are representative of four independent experiments.

activity was detected at 1 h in each group. These findings indicate that although MPF activity decreased prior to MAPK activity following egg activation in the absence of U0126 treatment, inactivation of MAPK occurred in parallel to inactivation of MPF under the inhibitory condition of the MAPK cascade.

Discussion

Activation of MAPK during oocyte maturation has been reported for mouse (Sobajima *et al*., 1993; Verlhac *et al*., 1993), porcine (Inoue *et al*., 1995), goat (Dedieu *et al*., 1996), rat (Zernicka-Goetz *et al*., 1997) and bovine (Fissore *et al*., 1996) oocytes. To our knowledge, however, there is no study reporting whether the elevated levels of MAPK activity in MII-arrested oocytes play a functional role in the subsequent M/G_1 transition in mammals. In the present study, when porcine oocytes matured *in vitro* were further cultured in medium containing the MEK inhibitor, U0126, the intensities of phosphorylated forms of ERK1/2 and the activities of MAPK and H1K were obviously reduced compared with those of oocytes cultured without U0126, and 24.3% of the oocytes underwent entry into interphase with PN formation (Figs. 2, 3). From these findings, it is clear that the decrease in MAPK activity is partly involved in driving matured oocytes out of metaphase to induce PN development in combination with MPF inactivation. Similarly, metaphase-arrested extracts from *Xenopus* eggs treated with nocodazole had high levels of MPF activity, failed to degrade cyclin B and contained an activated MAPK, and the addition of purified MAPK-specific phosphatase to these extracts resulted in a decline in H1K activity and entry into interphase (Minshull *et al*., 1994). Therefore, it is probable that MAPK activity before the onset of egg activation must be raised above a certain threshold level within the ooplasm in response to meiotic arrest at the MII stage.

In the present study, inactivation of MPF was found to occur very rapidly after electrostimulation and to correspond to initiation of meiotic resumption, whereas a long time sequence was required for dephosphorylation and inactivation of MAPK (Figs. 4, 6). This was correlated with PN formation in porcine oocytes without U0126 treatment, showing that MPF inactivation preceded MAPK inactivation after egg

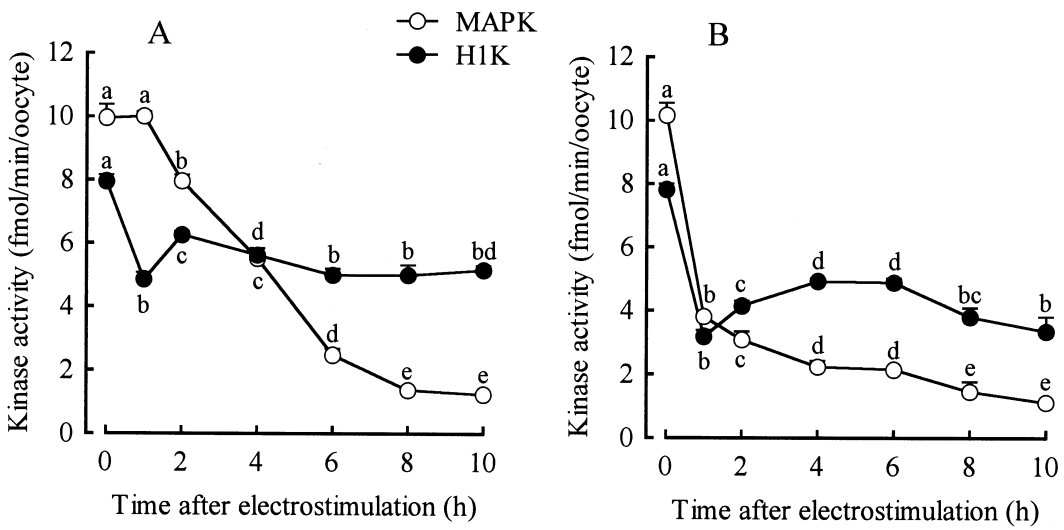

Figure 6 Time course changes in the activities of MAPK and H1K in porcine oocytes treated without (*A*) or with (*B*) 10 μM U0126 folowing electrostimulation. The values are expressed as mean ± SEM. [a–e]Within the same category, values with different superscripts are significantly different ($p < 0.05$).

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activation. Similar findings were found in *Xenopus* (Ferrell *et al*., 1991; Lorca *et al*., 1993), mouse (Verlhac *et al*., 1994; Moos *et al*., 1995), bovine (Liu *et al*., 1998, Liu and Yang, 1999) and porcine (Yamauchi *et al*., 1998) oocytes. The delayed inactivation of MAPK compared with MPF after egg activation will be reflected by the potent upstream functions of Mos, because cyclin subunits of MPF are degraded before Mos is degraded after fertilisation, and, thus, MPF activity is inactivated before CSF activity during activation of *Xenopus* oocytes (Watanabe *et al*., 1991). However, MAPK inactivation of oocytes treated with U0126 after electrostimulation coincided with inactivation of MPF, and PB-2 emission was strongly blocked in these oocytes (Table 1). The induction of egg activation without PB-2 emission has been reported previously in bovine oocytes co-treated with calcium ionophore A23187 and 6-dimethylaminopurine (Liu *et al*., 1998; Liu & Yang, 1999), and porcine oocytes treated with vanadate or olomoucine (Lee *et al*., 1999).

Gotoh *et al*. (1991) reported that MAPK could induce the interphase–metaphase transition of microtubule arrays in the cell extracts obtained from *Xenopus* oocytes. In mouse oocytes, MAPK is localised in microtubule organising centres and the meiotic spindle (Verlhac *et al*., 1993), and microtubule and chromatin behaviour is controlled by MAPK activity during meiosis (Verlhac *et al*., 1994). Furthermore, activation of the Mos/MAPK cascade in mouse oocytes leads to partial chromosome condensation and the formation of microtubule arrays under the inhibitory condition of MPF activation (Choi *et al*., 1996). In contrast, in c-*mos*-knockout mouse oocytes possessing very low levels of MAPK activity, the induction of germinal vesicle breakdown (GVBD) and PB-1 emission was similar to that in wild-type mouse oocytes, but large numbers of oocytes directly formed PN instead of undergoing second meiosis (Araki *et al*., 1996). This indicates that the Mos/MAPK cascade in mouse oocytes plays a crucial role in normal spindle and chromosome morphology after PB-1 emission. We observed that the addition of 10 μM U0126 to *in vitro* maturation medium did not inhibit GVBD and PB-1 emission during maturation of porcine oocytes, and that 82.8% (125/151) of oocytes reached the MII stage despite a significant decrease in the activity of MAPK (7.2 ± 0.5 fmol/min per oocyte) in comparison with the levels found in oocytes matured without U0126 (10.4 ± 0.5 fmol/min per oocyte) (unpublished observations). Taking these findings together, it is suggested that MAPK activity in porcine oocytes is closely associated with meiotic spindle formation to extrude PB-2 during egg activation, and that the delayed MAPK inactivation after the onset of MPF inactivation has an essential role in accomplishing the transition from meiosis to mitosis. It is unclear, however, why the emission of only PB-2, but not PB-1, is affected by low levels of MAPK activity. The anaphase-specific proteolysis of the *Schizosaccharomyces pombe* Cut2 protein, which has two sequences similar to the cyclin destruction boxes in the N-terminal region, is essential for sister chromatid separation (Funabiki *et al*., 1996). The functional differences of MAPK activity for regulating the emission of PB-1 and PB-2 will be the subject of further studies on the meiotic division.

Okadaic acid, a protein phosphatase inhibitor, was found to inhibit PN formation by preventing the decrease in MAPK activity in mouse oocytes. Thus, it is likely that MAPK inactivation was necessary for PN envelope assembly after fertilisation (Moos *et al*., 1995). Similarly, inactivation of MAPK after egg activation was associated with the formation of microtubular networks and PN development in bovine oocytes (Liu *et al*., 1998). In the present study, although the MAPK activity of oocytes treated with U0126 declined immediately after electrostimulation, PN formation in these oocytes was not accelerated in comparison with untreated oocytes. These finding suggest that MAPK activity may not be qualitatively essential for regulating the critical period necessary to undergo PN formation. Other factors are likely to be involved. Otherwise, remarkably low levels of MAPK activity may be indispensable for inducing PN formation, because the time course change in the intensities of phosphorylated forms of ERK1/2 after egg activation was not different, irrespective of U0126 treatment (Fig. 5).

In conclusion, the intensities of phosphorylated forms of ERK1/2 and the activities of MAPK and H1K in matured oocytes were significantly decreased by treatment with U0126, and these oocytes underwent entry into interphase with PN formation. Furthermore, the inactivation of MPF preceded that of MAPK in activated oocytes, but in oocytes treated with U0126 following electrostimulation, the decrease in MAPK activity was concomitant with MPF inactivation and the synchronous kinase inactivation elicited the inhibition of PB-2 emission. These findings suggest that the decrease in MAPK activity is partly involved in driving matured oocytes out of metaphase to induce PN development and that the delayed inactivation of MAPK after the onset of MPF inactivation plays an essential role in inducing PB-2 emission to accomplish the transition from meiosis to mitosis. Further studies are required to investigate whether other factors are involved in PB-2 emission and PN formation by associating with MAPK activity during egg activation.

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