Reprod Med Biol (2011) 10:89–96
DOI 10.1007/s12522-010-0073-2

ORIGINAL ARTICLE

# Effect of H89 on the meiotic resumption of pig oocytes

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Received: 5 November 2010 / Accepted: 21 December 2010 / Published online: 20 January 2011


**Abstract**
*Purpose* We studied the effect of H89, an inhibitor of protein kinase A (PKA), on the meiotic resumption of pig oocytes.
*Methods* Pig cumulus–oocyte complexes (COCs) and denuded oocytes (DOs) were cultured for 27 h to induce meiotic resumption. COCs and DOs were exposed to H89 for different periods. Oocyte PKA activity was assessed by in vitro kinase assay and immunocytochemistry using an antibody against fully active PKA catalytic subunits. Oocyte serine/threonine (Ser/Thr)-phosphorylated proteins were detected by Western blotting and immunocytochemistry using an anti-pSer/pThr PKA substrate antibody.
*Results* H89 suppressed germinal vesicle break down (GVBD) in COCs and DOs. To determine whether the suppression was due to inhibition of oocyte PKA, we analyzed oocyte PKA. Kinase assay showed that both types of oocytes possessed PKA activity throughout the culture period. Immunocytochemistry showed that fully active PKA catalytic subunits and Ser/Thr phosphorylated proteins were present in the oocytes at the GV stage and after GVBD. Western blotting indicated that both types of oocytes contained Ser/Thr phosphorylated proteins at the GV stage, and that several proteins became phosphorylated after GVBD.
*Conclusions* Pig oocytes contain active PKA during the occurrence of GVBD, and H89 suppresses the GVBD.

**Keywords** H89 · Oocyte maturation · Pig · PKA · Protein phosphorylation

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## Introduction

During oogenesis, mammalian oocytes acquire nuclear and cytoplasmic competence for future maturation and fertilization while arrested at the diplotene stage of the first meiotic division [1]. After gonadotropic stimulation, fully grown oocytes resume meiosis. During meiotic resumption, the chromosomes condense, the nuclear envelope breaks down (germinal vesicle break down or GVBD), the first meiotic spindle is formed and finally the cytoplasm of the oocyte divides asymmetrically to produce a polar body and a fertilizable "egg" [2].

Cyclic adenosine 3′,5′-monophosphate (cAMP) is a key regulator of the meiotic resumption of starfish, *Xenopus* and mammalian oocytes [3–5]. cAMP in the oocyte is thought to be derived from the follicular compartments, known as granulosa cells, which are connected to the oocyte through gap junctions. Also, the oocyte itself has been reported to synthesize cAMP, as demonstrated in mouse oocytes [2]. This level of intracellular cAMP allows the regulation of cascades involving meiotic resumption. A high level of the intracellular cAMP is related to the maintenance of the meiotic arrest, while a decrease of the level is related to meiotic resumption [6, 7].

Signals mediated by cAMP are mainly through protein kinase A (PKA), a heterotetramer consisting of two catalytic and two regulatory subunits. When the intracellular cAMP levels increase, the catalytic subunits are released from the holoenzyme and PKA becomes active [8]; in turn, PKA phosphorylates broad types of nuclear and cytoplasmic proteins at the serine and threonine position. Among these proteins, enzymes and transcription factors such as cAMP response element binding protein (CREB), which has been found in somatic cells, are included [9, 10].

The roles of PKA in the meiotic resumption of oocytes are controversial and the topic of ongoing discussion. In *Xenopus* oocytes, it was found that the inhibition of endogenous PKA, by the injection of the regulatory subunit of PKA or a peptide inhibitor, caused hormone-independent oocyte maturation. On the contrary, the injection of the catalytic subunit of PKA prevented progesterone-induced oocyte maturation [11]. However, it has been reported that the injection of catalytically inactive PKA also inhibited meiotic resumption [12]. In rodents' oocytes, cAMP analogues, which can activate PKA, maintain meiotic arrest, while in follicle enclosed oocytes, forskolin, which can arouse cAMP content, stimulates meiotic resumption [13, 14]. These observations suggest that cAMP signaling in the meiotic resumption of oocytes is apparent, but a clear picture of the implication of PKA has not yet been derived.

The time-dependent changes in cAMP levels in pig oocytes and cumulus–oocyte complexes (COCs) throughout the in vitro maturation have been reported previously [15, 16], but the activity of PKA during meiotic resumption remains unclear. In the present study, the effect of a PKA inhibitor *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89) on the meiotic resumption of pig oocytes in COCs and denuded oocytes (DOs) was studied. Since H89 suppressed the meiotic resumption and suppressed the GVBD of both types of oocytes, we investigated the idea that the activity of PKA was required for meiotic resumption. Considering the possible side effects or the non-specific action of H89, two additional experiments were conducted to show the presence of PKA activity and the phosphorylation of oocyte proteins by PKA during in vitro maturation.

## Materials and methods

### Chemicals and reagents

All the chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

### Oocyte collection and culture

Pig ovaries were obtained from prepubertal gilts at a local slaughterhouse. After three washes in Dulbecco's phosphate-buffered saline (PBS) containing 0.1% (w/v) polyvinyl alcohol (PVA; PBS-PVA), intact healthy antral follicles 4–6 mm in diameter were dissected in PBS-PVA from the ovaries. Follicles were opened in 25 mM HEPES-buffered medium 199 (HEPES-199; Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% (w/v) PVA, 10 mM sodium bicarbonate, and 0.08 mg/ml kanamycin sulphate, and COCs were isolated from the follicles and washed in HEPES-199. Since we considered the emergence of GVBD as the first evidence of meiotic resumption, the isolated COCs were matured in vitro up to 27 h in four-well multidishes (Nalge Nunc International, Tokyo, Japan), in 500 µl of basic maturation medium consisting of bicarbonate-buffered medium 199 supplemented with 10% (v/v) fetal calf serum (FCS; BioWhittaker, Walkersville, MD, USA), 0.1 mg/ml sodium pyruvate, 0.08 mg/ml kanamycin sulphate, and 0.1 IU/ml of human menopausal gonadotropin (hMG; Pergonal, Teikokuzoki, Tokyo, Japan), in an atmosphere of 5% $CO_2$ in humidified air at 38.5°C. For maturation of DOs, cumulus cells were removed from oocytes by gentle pipetting with a small-bored pipette in HEPES-199. Maturation medium for DOs was the same as for COCs but without gonadotropic hormone supplementation.

A group of 10–15 COCs and DOs each were maturation-cultured for six different periods: 3, 6, 15, 18, 24 and 27 h. Different groups of COCs were collected separately soon after removal from the follicular compartment (named as 0 h), denuded, fixed and stained as described below. After culture of COCs, oocytes were denuded by treatment with 0.05% (w/v) hyaluronidase (type I–S) and repeated pipetting with a small-bored pipette, mounted on slides, fixed with acetic acid–ethanol (1:3, v/v) solution, and stained with 1% (w/v) aceto-orcein. DOs were fixed and stained in the same manner. Stage of oocyte maturation was determined following the classification as described previously [17]. Oocytes showing abnormal distribution of chromosomes in the cytoplasm or signs of cytoplasmic degeneration were classified as degenerated oocytes.

### H89 treatment

H89 (Upstate Cell Solution, Charlottesville, VA, USA) competes for the ATP-binding site of the PKA catalytic subunit, inhibiting its action. H89 is widely used at concentrations ranging from 10 to 100 µM in somatic cells and *Xenopus* oocytes, and its effective dose varies among the cell types [18]. Pig oocytes degenerated when they were exposed to concentrations higher than 50 µM for a prolonged time. Therefore, we employed a final concentration of 25 µM in the maturation medium. H89 was dissolved in dimethylsulfoxide (DMSO; Wako Pure Chemical Industries, Osaka, Japan) and store at 4°C.

To analyze the effect of H89 on the resumption of meiosis, we cultured 10–15 COCs in the maturation medium for 18 and 21 h each and then transferred the samples to the H89-containing medium to complete 27 h of culture. In the same way, 10–15 DOs were cultured for 6, 15, 18 and 24 h, separately in the maturation medium and then

transferred to the H89-containing medium, to complete 27 h of culture. Two control groups were not subjected to H89 treatment but one of them was cultured in a medium containing 0.025% (v/v) DMSO as the vehicle.

### Kinase activity assay

PKA substrate peptide (DLDVPIPGRFDRRVSVAAE [Ala97]-RII (81–99); Biomol International, Plymouth Meeting, PA, USA) was used as a substrate to determine the activity of PKA by in vitro kinase activity assay of oocytes from COCs and DOs. Samples consisting of three oocytes from COCs or DOs were collected at 0 h and after 3, 6, 15, 18, 24 and 27 h of in vitro maturation. For each sample, the oocytes were denuded, washed in PBS-PVA and transferred into an Eppendorf tube with 1 µl of PBS-PVA. Then 4 µl of ice-cold extraction buffer was added and kept at −80°C until kinase assay. The extraction buffer was composed of 25 mM HEPES (pH 7.2), 80 mM $\beta$-glycerophosphate, 15 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA), 1 mM APMSF (Wako), 0.1 mM $Na_3VO_4$, 1 µg/ml leupeptin and 1 µg/ml aprotinin [19].

After thawing, samples were centrifuged at 12500×*g* for 2 min at 2°C, supplemented with 5 µl of kinase buffer and 5 µl of 0.25 mg/ml of PKA substrate peptide, and incubated for 20 min at 37°C. The kinase buffer was composed of 75 mM HEPES (pH 7.2), 75 mM $\beta$-glycerophosphate, 75 mM $MgCl_2$, 6 mM DTT, 10 mM EGTA, 60 µM ATP and 0.3 µCi/µl [$\gamma$-$^{32}$P]ATP (250 µCi/25 µl; GE Healthcare UK, Buckinghamshire, England). To discriminate PKA specific activity, 15 µg/ml of PKA inhibitor peptide (PKI sequence: TTYADFIASGRTGRRNAIHD) was added to the kinase buffer. Reaction was stopped by adding 5 µl of quadruple-strength-concentrated SDS sample buffer [20] and boiling for 5 min. Samples were loaded onto a 16% SDS-polyacrylamide-glycerol gel for separation of labeled PKA substrate peptide in Tris–Tricine buffer system. After running, gels were dried and autoradiographed.

### SDS-PAGE and immunoblotting

For the detection of serine/threonine phosphorylated PKA substrates, groups of 60 oocytes each were collected at 0 h and after 3, 6, 15, 18, 24 and 27 h of culture. They were boiled for 5 min in 15 µl of double-strength-concentrated SDS sample buffer and kept at −20°C until use. Oocyte proteins were separated by SDS-PAGE using a 12.5% gel and then transferred electrophoretically to polyvinylidene fluoride membrane (Immobilon P, Millipore, Bedford, MA, USA). The membrane was blocked in PBS containing 0.1% (v/v) Tween-20 (PBS-Tween-20; Wako) and 10% FCS (v/v) (Dainippon Sumitomo Pharma, Osaka, Japan) for 1 h at room temperature, incubated for 3 h in the primary antibody rabbit polyclonal anti-phosphoserine/phosphothreonine PKA antibody (Cell Signaling Technology, Danvers, MA, USA, Cat.#9621 named pS/pT PKA substrate antibody, 1:500 in PBS-Tween-20 containing 5% FCS) followed by three washes in PBS-Tween-20, blocked for 30 min in PBS-Tween-20 containing 10% FCS, and incubated with horseradish peroxidase conjugated donkey anti-rabbit immunoglobulins (GE Healthcare UK, Cat.#-NA934V, 1:1000) in the blocking buffer for 90 min. After washing three times, peroxidase activity was visualized using Western Blotting Luminol Reagent (Chemi-Lumi One, Nacalai Tesque, Kyoto, Japan) and Hyperfilm-ECL (GE Healthcare UK).

### Fluorescence microscopy

To elucidate the localization of active PKA, oocytes from COCs and DOs were examined at 0 h and after 27 h of in vitro maturation by immunofluorescence microscopy. After being washed twice in PBS-PVA, oocytes were fixed in 4% (w/v) paraformaldehyde in PBS-PVA containing 0.2% (v/v) Triton X-100 (Nacalai Tesque) for 40 min. Fixed oocytes were washed twice in PBS-PVA for 15 min each, then stored in PBS-PVA containing 10 mg/ml bovine serum albumin (BSA; Wako) (BSA-PBS-PVA) at 4°C overnight. The oocytes were incubated with primary antibody, rabbit anti-phospho-PKA C (Thr197) antibody [Cell Signaling Technology, Cat.#4781 named phospho-PKA C (Thr197) antibody, 1:50 in BSA-PBS-PVA] at 4°C overnight. After washing three times in BSA-PBS-PVA for 15 min each, oocytes were reacted with Alexa Fluor 488-labeled goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA, Cat.#A11008, 1:400) for 40 min at room temperature. Next, oocytes were washed three times in BSA-PBS-PVA for 15 min each, and DNA was counterstained with 14.3 µM 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) for 20 min at 4°C. The oocytes were washed three times in BSA-PBS-PVA before being mounted on glass slides with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA), and observed under fluorescence microscope (IX70-S1F2; Olympus Optical, Tokyo, Japan). A different group of oocytes were treated with anti-pS/pT PKA substrate antibody (1:50 in BSA-PBS-PVA) followed by the same procedures as mentioned above.

### Statistical analysis

The frequencies of oocytes at each stage of meiotic maturation were analyzed by the chi-square test. *P* values lower than 0.05 were considered to indicate statistical

significance. Each experiment at every period was conducted at least three times and the data were pooled and analyzed.

## Results

### Meiotic resumption of oocytes

The time courses of meiotic resumption of oocytes in COCs and DOs were different from each other. At 24 h after gonadotropic stimulation, 81% of the oocytes in COCs underwent GVBD and 72% were at metaphase I (Table 1). After 27 h, GVBD oocytes increased to 87%; meanwhile, 81% of oocytes reached metaphase I. DOs resumed meiosis rapidly compared to oocytes in COCs in our culture conditions. After 15 h of culture, 36% of DOs underwent GVBD and 16% reached metaphase I. Thereafter, the number of GVBD-DOs increased gradually up to 27 h, where 83% of oocytes underwent GVBD and 19% reached metaphase II.

To examine the effect of H89 on the meiotic resumption, COCs and DOs were cultured in the maturation medium and then transferred to the medium containing 25 μM of H89 for a given time (Table 2). Meiotic resumption in oocytes in COCs and DOs was suppressed as they were exposed longer in H89-containing medium. In COCs, 100% of the oocytes underwent GVBD and 70% reached metaphase I at 27 h of culture, but when they were transferred during the last 6 h to H89-containing medium, the rate of GVBD decreased to 78% and no oocyte reached metaphase I. Also, when they were transferred during the last 9 h of culture to H89-containing medium, only 7% resulted in GVBD. In the same way, the exposure of DOs cultured for 6 h to H89-containing medium decreased the GVBD rate to 29%. Conversely, the exposure of DOs cultured for 18 h or beyond to H89-containing medium did not affect GVBD significantly. This suggests that H89 suppresses the appearance of oocyte GVBD.

### PKA activity during meiotic resumption of oocytes

The activity of PKA was elucidated by kinase activity assay. Figure 1A shows PKA activity of oocytes from COCs and DOs during 27 h of in vitro maturation. Phosphorylation of the PKA substrate peptide by the extracts of oocytes from COCs was detected strongly around 3–6 h and in lesser intensity during 15–27 h of culture (Fig. 1Aa). Phosphorylation throughout the in vitro maturation decreased considerably when PKI was added to the kinase assay mixture (0–27 h) (Fig. 1Ab), suggesting that the phosphorylation was caused by active PKA. In contrast, the phosphorylation of the PKA substrate peptide by the DO extracts was relatively constant throughout the maturation time-points (Fig. 1Ac), and the activity was again lessened by adding PKI (Fig. 1Ad).

Since Thr197 phosphorylation is important for the full activation of the catalytic subunit of PKA [21], we performed immunofluorescence microscopy using anti-

**Table 1** Maturation stage of pig oocytes cultured in vitro

| Oocytes | Culture time (h) | No. of oocytes examined | No. (%) of oocytes at GV stage | No. (%) of oocytes after GVBD | | | | | No. (%) of degenerated oocytes |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Total GVBD | D | MI | AI–TI | MII | |
| COCs | 0 | 50 | 50 (100) | 0 | 0 | 0 | 0 | 0 | 0 |
| | 3 | 50 | 50 (100) | 0 | 0 | 0 | 0 | 0 | 0 |
| | 6 | 45 | 45 (100) | 0 | 0 | 0 | 0 | 0 | 0 |
| | 15 | 48 | 45 (94)$^{a}$ | 0 | 0 | 0 | 0 | 0 | 3 (6) |
| | 18 | 54 | 54 (100) | 0 | 0 | 0 | 0 | 0 | 0 |
| | 24 | 53 | 10 (19)$^{bc}$ | 43 (81)$^{ab}$ | 5 (9) | 38 (72)$^{a}$ | 0 | 0 | 0 |
| | 27 | 54 | 3 (6)$^{c}$ | 47 (87)$^{a}$ | 3 (6) | 44 (81)$^{a}$ | 0 | 0 | 4 (7) |
| DOs | 0 | 45 | 45 (100) | 0 | 0 | 0 | 0 | 0 | 0 |
| | 3 | 50 | 50 (100) | 0 | 0 | 0 | 0 | 0 | 0 |
| | 6 | 48 | 48 (100) | 0 | 0 | 0 | 0 | 0 | 0 |
| | 15 | 44 | 26 (59)$^{d}$ | 16 (36)$^{c}$ | 9 (20) | 7 (16)$^{b}$ | 0 | 0 | 2 (5) |
| | 18 | 47 | 25 (54)$^{de}$ | 19 (40)$^{c}$ | 8 (17) | 11 (23)$^{bc}$ | 0 | 0 | 3 (6) |
| | 24 | 44 | 16 (37)$^{be}$ | 28 (63)$^{b}$ | 2 (5) | 16 (36)$^{bc}$ | 5 (11) | 5 (11) | 0 |
| | 27 | 47 | 6 (13)$^{c}$ | 39 (83)$^{a}$ | 6 (13) | 18 (38)$^{c}$ | 6 (13) | 9 (19) | 2 (4) |

Values with different superscripts in the same column differ significantly ($P < 0.05$). Data at each point of culture time represent the total number of three replicates

*COCs* cumulus–oocyte complexes, *DOs* denuded oocytes, *GV* germinal vesicle stage, *GVBD* germinal vesicle break down, *D* diakinesis, *MI* metaphase I, *AI–TI* anaphase I–telophase I, *MII* metaphase II

**Table 2** Effect of H89 on the maturation of pig oocytes cultured in vitro

| Oocytes | Culture time (h)[A] | | No. of oocytes examined | No. (%) of oocytes at GV stage | No. (%) of oocytes after GVBD | | | | | No. (%) of degenerated oocytes |
|---|---|---|---|---|---|---|---|---|---|---|
| | Medium | H89 | | | Total GVBD | D | MI | AI–TI | MII | |
| COCs | 0 | 27 | 48 | 32 (67)$^{a}$ | 0 | 0 | 0 | 0 | 0 | 16 (33)$^{a}$ |
| | 18 | 9 | 45 | 42 (93)$^{b}$ | 3 (7)$^{a}$ | 3 (7)$^{a}$ | 0 | 0 | 0 | 0 |
| | 21 | 6 | 45 | 10 (22)$^{cd}$ | 35 (78)$^{b}$ | 35 (78)$^{b}$ | 0 | 0 | 0 | 0 |
| | 27 | 0 | 46 | 0 | 46 (100) | 14 (30)$^{c}$ | 32 (70)$^{a}$ | 0 | 0 | 0 |
| In DMSO[B] | 27 | 0 | 44 | 3 (7)$^{d}$ | 39 (88)$^{b}$ | 4 (9)$^{ad}$ | 35 (79)$^{a}$ | 0 | 0 | 2 (5)$^{b}$ |
| DOs | 0 | 27 | 45 | 13 (29)$^{cd}$ | 6 (13)$^{ac}$ | 6 (13)$^{acd}$ | 0 | 0 | 0 | 26 (58)$^{c}$ |
| | 6 | 21 | 45 | 19 (42)$^{c}$ | 13 (29)$^{c}$ | 13 (29)$^{c}$ | 0 | 0 | 0 | 13 (29)$^{a}$ |
| | 15 | 12 | 46 | 15 (33)$^{cd}$ | 15 (33)$^{c}$ | 11 (24)$^{cd}$ | 4 (9)$^{bc}$ | 0 | 0 | 16 (34)$^{a}$ |
| | 18 | 9 | 46 | 0 | 35 (76)$^{b}$ | 11 (24)$^{cd}$ | 4 (9)$^{bc}$ | 8 (17) | 12 (26)$^{a}$ | 11 (24)$^{a}$ |
| | 24 | 3 | 45 | 0 | 37 (82)$^{b}$ | 11 (24)$^{cd}$ | 9 (20)$^{c}$ | 0 | 17 (38)$^{ab}$ | 8 (18)$^{ab}$ |
| | 27 | 0 | 45 | 0 | 37 (82)$^{b}$ | 0 | 11 (24)$^{cd}$ | 0 | 26 (58)$^{b}$ | 8 (18)$^{ab}$ |
| In DMSO[B] | 27 | 0 | 54 | 8 (15)$^{d}$ | 46 (85)$^{b}$ | 5 (9)$^{ad}$ | 22 (41)$^{d}$ | 9 (17) | 10 (19)$^{a}$ | 0 |

Values with different lowercase superscripts in the same column differ significantly ($P < 0.05$). Data at each point of culture time represent the total number of three replicates

*GV* germinal vesicle stage, *GVBD* germinal vesicle break down, *D* diakinesis, *MI* metaphase I, *AI–TI* anaphase I–telophase I, *MII* metaphase II

[A] Cumulus–oocyte complexes (COCs) and denuded oocytes (DOs) were cultured for 27 h. First they were cultured in the maturation medium for the given culture time in the second column (medium) and then transferred to 25 μM H89-containing medium (H89) to complete 27 h

[B] DMSO: H89 dissolved in dimethylsulfoxide (DMSO) as the vehicle was added to the culture medium to give the final concentration of 25 μM [concentration of DMSO in the medium: 0.025% (v/v)]. A control group was cultured for 27 h in the medium with a final concentration of 0.025% (v/v) DMSO

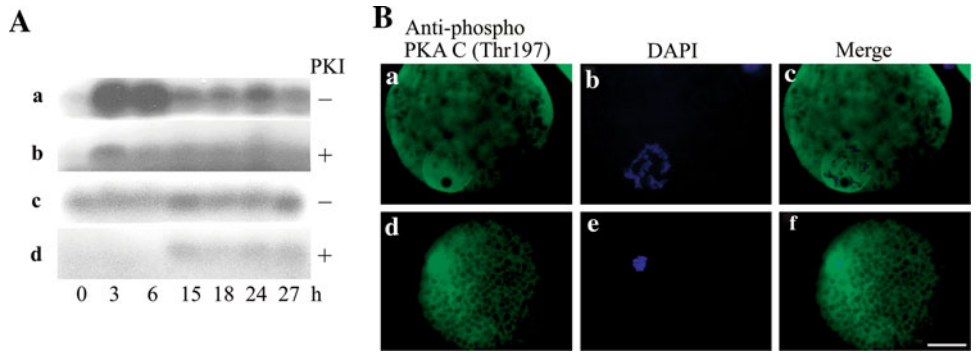


**Fig. 1** PKA activity during meiotic resumption of pig oocytes. **A** Changes in protein kinase A (PKA) activity of pig oocytes during meiotic maturation. COCs and DOs were cultured for various time periods indicated in the lower row. PKA activities of three oocytes from COCs (*a*, *b*) or DOs (*c*, *d*) per lane were measured by the phosphorylation of PKA substrate peptide as the substrate. A PKA peptide inhibitor (PKI) was added to the reaction mixture (*b*, *d*) to determine the specificity of PKA activity. One result of three separate experiments is shown here. **B** Localization of active PKA catalytic subunit in pig oocytes from COCs. Immunofluorescent staining was performed on oocytes with anti-phospho-PKA catalytic subunit (Thr197) antibody followed by Alexa Fluor 488-labeled secondary antibody (*green*) at 0 h (*a–c*) and after culture for 27 h (*d–f*). DAPI staining marks chromatin in *blue*. *Scale bar* 30 μm

phospho PKA C (Thr197) antibody. Localization was similar in oocytes from COCs and DOs, and the results of oocytes from COCs are shown in Fig. 1B. In GV oocytes, the phosphorylated catalytic subunit was localized in the cytoplasm and the GV at 0 h (7/10, Fig. 1Ba–c). In GVBD oocytes, after 27 h of in vitro maturation, it was localized in the cytoplasm and the region surrounding the condensed chromosomes in oocytes from COCs (15/15, Fig. 1Bd–f) and DOs (10/10, data not shown).

## Phosphorylation of oocyte proteins by PKA

Phosphorylation of oocyte proteins by PKA was studied with the anti-pS/pT PKA substrate antibody (Fig. 2). The time-dependent changes in the protein phosphorylation state in oocytes from COCs and DOs are shown in Fig. 2A, B, respectively. Throughout the culture, oocytes from COCs and DOs contained phosphorylated proteins. Oocytes proteins of 250, 110, 59 and 42 kDa became

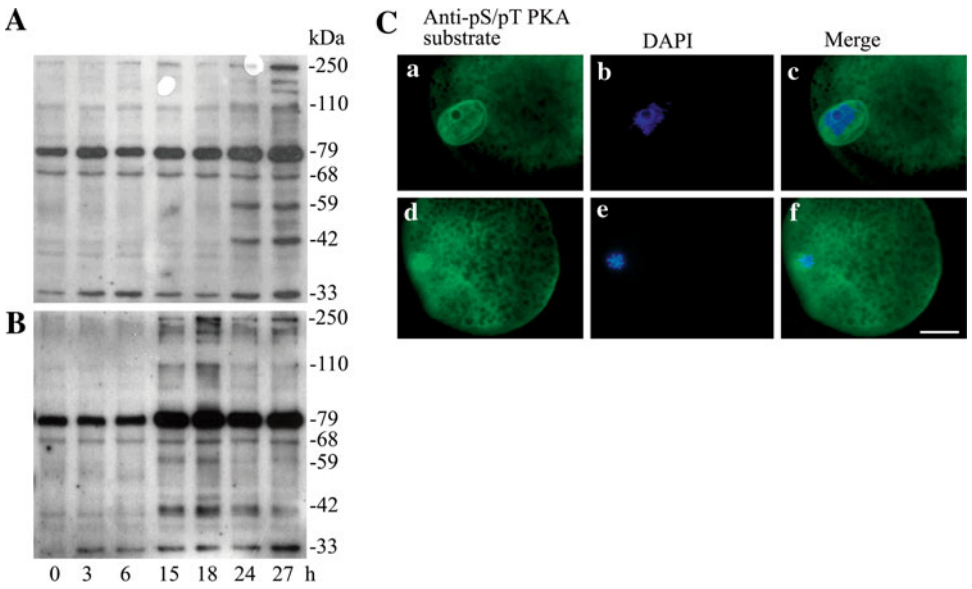


**Fig. 2** Time-related changes of phosphorylated proteins at serine and threonine residues in pig oocytes from COCs (**A**) and DOs (**B**). Sixty oocytes per lane were collected at 0 h and after culture at every time period (3–27 h) given in the lower row and Western blotted with anti-phospho serine/threonine (anti-pS/pT) PKA substrate antibody followed by horseradish peroxidase-conjugated secondary antibody. *Each blot* represents one of three replicates. **C** Localization of phosphorylated proteins at serine and threonine residues by PKA in pig oocytes from COCs. Immunofluorescent staining was performed on oocytes with anti-pS/pT PKA substrate antibody followed by Alexa Fluor 488-labeled secondary antibody (*green*) at 0 h (*a–c*) and after culture for 27 h (*d–f*). DAPI staining marks chromatin in *blue*. *Scale bar* 30 μm

phosphorylated at approximately the timing of GVBD in oocytes from COCs (Fig. 2A) and DOs (Fig. 2B) at 24 and 15–18 h, respectively. A 68 kDa protein was phosphorylated throughout the culture in both types of oocytes. In addition, 79 and 33 kDa phosphorylated proteins increased at the timing of enhancement of PKA activity in oocytes in COCs (3–6 h) (Figs. 1A, 2A).

We used the same antibody to localize the proteins phosphorylated at serine and threonine residues in oocytes (Fig. 2C). In GV oocytes at 0 h (Fig. 2Ca–c), the phosphorylated proteins were localized in the cytoplasm and the GV (15/15). In GVBD oocytes after 27 h culture (Fig. 2Cd–f), they were localized in the cytoplasm and the region surrounding the condensed chromosomes of oocytes from COCs (15/15) and DOs (10/10, data not shown).

## Discussion

During in vitro maturation, DOs underwent GVBD earlier than did oocytes in COCs. Moreover, DOs reached MII sooner than oocytes in COCs. This suggests that meiotic progression is faster in DOs compared to oocytes in COCs. The difference between these two experimental models (DOs and oocytes in COCs) is the presence of cumulus cells. It is known that cumulus cells have inhibitory effects on the appearance of GVBD through cAMP, which is synthesized by cumulus cells and transferred to the oocytes through gap junctions; this, in turn, results in maintenance of the meiotic arrest at the GV stage [2]. Therefore, in DOs, the oocytes were released from the inhibitory effect of the cumulus cells and resumed meiosis spontaneously at an earlier time than oocytes in COCs.

In both COCs and DOs, H89 suppressed the meiotic resumption of oocytes, and the inhibitory effect was stronger in oocytes in COCs than in DOs. Assuming that H89 inhibits PKA, it seems likely that PKA activity is not only involved in the meiotic arrest [11, 12] but also in the meiotic resumption of oocytes, especially those in COCs. It was recently reported, that the inhibition of PKA by H89 during the first hours after hormonal stimulation downregulated the gene expression of EGF-like factor and TACE/ADAM 17 in cumulus cells, which are involved in oocyte maturation [22, 23]. Therefore, H89 may also have inhibited meiotic resumption in this way in COCs in the present study. The involvement of PKA in multiple cascades, such as the activation of CREB for protein synthesis [9, 10] and the phosphorylation of histones for chromatin remodeling [24] have also been reported in somatic cells. Therefore, H89 possibly affected these pathways in the oocytes via PKA inhibition. However, since the pharmacological and toxicological properties of H89 have not been fully investigated, a possible side effect of H89 to the oocytes needs to be considered. In addition, since H89 action competes for the ATP-binding sites of PKA, a non-specific reaction to other kinases

should also be considered [25]. In this research, the possibility that PKA activity is required for meiotic resumption of oocytes is contemplated.

Kinase assay showed that both types, oocytes in COCs and DOs, had PKA activity throughout the culture. A transient increase in PKA activity was observed in oocytes in COCs at 3–6 h, while PKA activity in DOs was low and constant during 27 h of in vitro maturation. The transient increase in PKA activity of oocytes in COCs was similar to the changes in cAMP content reported in pig oocytes. After hormonal stimulation, the level of cAMP increases in oocytes in COCs from 6 to 15 h and thereafter starts to decrease until it becomes low and constant after 24 h [15, 16]. Studies done in rodents demonstrate that after hormonal stimulation cAMP from cumulus cells passes through gap junctions to the oocyte [26, 27], activating PKA in the oocyte [28]. Since DOs did not show any increase in PKA activity throughout the in vitro maturation culture, the increase of PKA activity (3–6 h) observed in oocytes in COCs could be due to the increment of cAMP in the oocytes that comes from cumulus cells [27, 29]. The effect of H89 on GVBD depended on the time of its exposure. H89 suppressed GVBD in oocytes in COCs and DOs when exposed for 18–27 and 15–27 h, but not 21–27 and 18–27 h, respectively. This means that oocytes in COCs and DOs were suscepible to the effect of H89 around 18–21 and 15–18 h, respectively. Since, both types of oocytes underwent GVBD around these times (18–21 and 15–18 h) and pig oocytes themselves are able to produce cAMP throughout the maturation [15, 16, 30], basal PKA activity during these times may be required for GVBD of oocytes. On the other hand, the observed peak of PKA activity in oocytes in COCs (3–6 h) may be involved in the maintenance of temporal meiotic arrest to synchronize the timing of GVBD of oocytes in COCs, rather than being involved in oocyte GVBD.

In immunofluorescent study, active PKA was detected in the oocytes at both the GV stage and after GVBD. An antibody used in this study recognizes the PKA catalytic subunits only when they are phosphorylated at the Thr197 position and become fully active. It was reported that PKA localization differs according to their isoform types: while type I isoform is diffused in the cytoplasm, type II is localized in cellular organelles [31]. The results found here are consistent with the results of previous reports, where the catalytic subunit of PKA in rat growing oocytes was distributed homogenously in the cytoplasm [32]. Similar results were found in mouse oocytes, where the catalytic subunit of PKA was found in the GV and the cytoplasm of GV oocytes and the cytoplasm of GVBD oocytes [33]. These results suggest that pig oocytes have a basal PKA activity during the GV stage and after GVBD.

The antibody against serine/threonine phosphorylated substrates by PKA used in this study identifies substrates of the AGC family kinases, which include PKA, PKG and PKC. This antibody detected proteins phosphorylated at the serine/threonine position during meiotic resumption of pig oocytes. In Western blotting, several bands were phosphorylated in the oocytes before in vitro maturation. Recently, using an anti-pS/pT PKA substrate antibody and mass-spectrometry, ribosomal protein S6, EF-2, poly A binding protein and releasing factor-1 were identified as PKA targets in *Xenopus* GV oocytes [34]. In the present study, in oocytes from COCs, only 79 and 33 kDa bands increased slightly at the same period when the transient increase of PKA activity was found by kinase assay (3–6 h). In addition, 250, 110, 59 and 42 kDa bands were phosphorylated at the time of GVBD in oocytes from COCs and DOs. These results are in agreement with the reports showing that the phosphorylation of several proteins increases at the time of GVBD involving the participation of CDK1 and MAP kinase, both of which activate many other downstream kinases [35–37]. As mentioned above, both oocytes in COCs and DOs were susceptible to the inhibitory effect of H89 at the time of GVBD (18–21 and 15–18 h, respectively) but after these times, H89 had no effect on GVBD at all. Assuming that H89 affects PKA activity, the results found in this study suggest that once PKA phosphorylates specific substrate proteins and GVBD occurs, the progression of oocyte maturation might become independent or separate from PKA. The phosphorylated substrates were localized in the cytoplasm and GV of pig GV oocytes and around the chromosome area and the cytoplasm of GVBD oocytes. The localization of these substrates was similar to the localization of phosphorylated PKA catalytic subunit. This co-localization may indicate a possible role in the phosphorylation of these substrates by PKA.

In conclusion, the results of the present study suggest that H89 suppresses the GVBD of pig oocytes possibly through the inhibition of oocyte basal PKA activity.

**Acknowledgments** We express our considerable gratitude to the staff of the Kobe Meat Inspection Office for supplying pig ovaries. This research was supported in part by a Grant-in-Aid for Scientific Research of the Japan Society for the Promotion of Science to T.M.

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