Meiotic Resumption of Pig Oocytes by cAMP-Dependent Protein Kinase Inhibitor H89

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Abstract: *There is abundant evidence that cAMP plays an important role in maintaining meiotic arrest in mammalian oocytes. In mouse oocytes, cAMP and cAMP-dependent protein kinase (PKA) form part of a negative pathway which participates in the maintenance of meiotic arrest. In the present experiment, involvement of PKA on the meiotic arrest of pig oocytes was examined using PKA specific inhibitor H89. Cumulus-enclosed oocytes with or without parietal granulosa tissue were incubated in H89-supplemented medium for various durations, and subsequently cultured in H89-free medium for a total of 24 hr. Over 95% of both types of oocytes were arrested at the germinal vesicle stage after 24 hr in H89-free medium. On the other hand, after H89 treatment, about 40% (75 μM H89 for 6 hr, and 100 μM for 4 hr) of cumulus-enclosed oocytes with parietal granulosa tissue and about 70% (50 μM for 6 hr, and 75 μM for 4 hr) of cumulus-enclosed oocytes without granulosa tissue underwent germinal vesicle breakdown (GVBD), respectively. Pig denuded oocytes released from granulosa cells resumed meiosis spontaneously (75% GVBD), but the spontaneous resumption was inhibited by a component of pig follicular fluid, hypoxanthine (12% GVBD). Under the inhibition of hypoxanthine, 42% of denuded oocytes underwent GVBD after treatment with 100 μM H89 for 30 min. These results suggest PKA involvement in the meiotic arrest of pig oocytes.*

Key words: *H89, Hypoxanthine, Meiotic arrest, Oocyte, Pig, PKA*

Fully grown mammalian oocytes undergo hormone-independent resumption of the first meiotic division spontaneously in culture [1, 2]. There is abundant evidence that cyclic 3', 5'-adenosine monophosphate (cAMP) plays an important role in maintaining meiotic arrest in fully grown oocytes. Spontaneous meiotic resumption of oocytes is prevented by the agents which sustain the elevated cAMP levels in oocytes of several mammalian species. Such agents include a cAMP analog, dibutyryl cAMP (dbcAMP) [3–8], an adenylate cyclase activator, forskolin [9–14], and a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) [15, 16]. During the meiotic resumption, decrease of cAMP levels occurs in starfish [17], *Xenopus* [18] and mouse oocytes [19]. Moreover, the meiotic resumption is induced through injection of phosphodiesterases into oocytes of *Xenopus* [20] and mouse [21].

cAMP-dependent protein kinase (PKA) is the principle mediator of cAMP. In the absence of cAMP, PKA exists as a tetramer composed of two catalytic subunits (C subunits) and two regulatory subunits (R subunits) [22]. In the holoenzyme form, PKA is inactive, and it is activated by binding of cAMP to the R subunits. cAMP allows the release of active C subunits from the R subunits, and the free C subunits can then catalyze the phosphorylation of specific cellular proteins on serine and threonine residues. In *Xenopus* and mouse oocytes, PKA has been proposed as a mediator of maintenance of meiotic arrest by cAMP [21, 23]. Microinjection of either inhibitor protein of PKA or R subunits induces meiotic resumption in *Xenopus* oocytes, while C subunit inhibits hormone-induced meiotic resumption [23, 24]. Similarly, in mouse oocytes, microinjection of inhibitor protein of PKA induces meiotic resumption in the presence of either dbcAMP or IBMX, while C subunit inhibits the resumption [21].

A potent and selective inhibitor of PKA, H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide), was synthesized by Chijiwa *et al.* [25]. H89 little inhibits other kinases, such as cGMP-dependent protein kinase (protein kinase G), Ca^{2+}/phospholipid-dependent protein

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kinase (protein kinase C), casein kinase I and II, myosin light chain kinase, and Ca^{2+}/calmodulin-dependent protein kinase II, but it directly inhibits PKA without any effects on synthesis or degradation of cAMP in PC12 pheochromocytoma [25]. Kinetic analysis indicates that H89 inhibits PKA in a competitive fashion against ATP [25]. Recently, Rose-Hellekant and Bavister reported that H89 interfered with forskolin or IBMX arrested bovine oocytes in a dose dependent manner [26], and that administration of H89 into hamsters induced meiotic resumption of ovarian oocytes [27]. These results show that PKA may form part of a negative pathway which participates in the maintenance of meiotic arrest in mammalian oocytes other than mice. In the present study, we examined the effect of H89 on meiotic arrest in pig oocytes.

Materials and Methods

Collection of pig oocytes

Pig ovaries were obtained from prepubertal gilts at a local slaughterhouse. Following three washes in Dulbecco's phosphate-buffered saline containing 0.1% polyvinyl alcohol (PBS-PVA), healthy antral follicles of 4–6 mm in diameter were dissected in PBS-PVA from ovaries following the technique described by Moor and Trounson [28]. After the follicles were opened in 25 mM HEPES-buffered medium 199 (Earle's salt, Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing glutamine and 0.08 mg/ml kanamycin sulphate (Sigma, St. Louis, MO), oocytes surrounded by cumulus cells with a piece of parietal granulosa tissue (OCGs) and oocytes with cumulus cells (OCs) were isolated from the follicles [29]. Denuded oocytes were obtained by mechanically removing cumulus cells from OCs with a small-bore pipette in a medium containing 6 mM hypoxanthine (Kohjin Co. Ltd., Tokyo, Japan).

Oocyte culture and examination

Culture medium was bicarbonate-buffered medium 199 supplemented with 10% fetal calf serum (Biocell Co. Ltd., Carson, CA), 0.1 mg/ml sodium pyruvate, and 0.08 mg/ml kanamycin sulphate. H89 (Biomol Research Labs., Inc., Plymouth Meeting, PA) was dissolved in 50% ethanol at various concentrations so that addition of 2.5 μl of it into 0.5 ml of the culture medium just before use made final concentrations of 0 (control), 50, 75 or 100 μM. Following two washes in culture media, OCGs or OCs were transferred into 0.5 ml of the culture medium containing H89 and cultured for 2, 4, 6 or 24 hr. Then they were washed in H89-free medium, and further cultured up to 24 hr in H89-free medium. Denuded oocytes were cultured in the culture medium supplemented with 6 mM hypoxanthine for prevention of spontaneous meiotic resumption. They were treated with 100 μM H89 for 10 min, 30 min, 1 or 2 hr, and subsequently cultured in H89-free, 6 mM hypoxanthine-supplemented medium up to 24 hr. Culture was carried out in an atmosphere of 5% CO_2 in humidified air at 38.5°C.

All of the oocytes after culture were denuded with a fine pipette, fixed with acetic acid and ethanol (1:3, v/v), and stained with 1% aceto-orcein. They were examined with Nomarski interference microscopy to determine the stage of meiosis. Oocytes with disintegration of the nuclear membrane (germinal vesicle breakdown, GVBD) were counted as having resumed meiosis. Oocytes showing abnormal cytoplasmic morphology were considered to be degenerating oocytes.

Statistical analysis

Data shown were pooled from at least three experiments. Statistical differences of the numbers of oocytes undergoing GVBD and degenerating were analyzed by chi-square test and Fisher's exact test. A P value less than 0.05 was considered statistically significant.

Results

Table 1 shows that H89 induced GVBD of pig OCGs. After 24 hr 98% of oocytes cultured in H89-free medium remained at the GV stage. The percentages of oocytes that had undergone GVBD were significantly higher when the oocytes were treated with 50 μM H89 for 4 and 6 hr. At concentrations of 75 and 100 μM, H89 treatment for 2 hr was effective for GVBD as well as 4 and 6 hr. However, the number of degenerating oocytes increased when they were treated with high concentrations of H89.

H89 also induced GVBD of pig OCs (Table 2). After 24 hr, 95% of oocytes cultured in H89-free medium remained at the GV stage. The percentages of oocytes that had undergone GVBD were significantly higher when the oocytes were treated with 50 μM H89 for 4 and 6 hr (50% and 71%, respectively). As the concentration of H89 was increased, oocytes underwent GVBD after short treatment of H89. At a concentration of 100 μM, H89 treatment for 2 hr was most effective for GVBD and 21% of the oocytes reached metaphase II. However, the number of degenerating oocytes increased when they were treated with high concentrations of H89, and more than half of the oocytes treated with H89 for 24 hr were degenerated.

Table 1. Effect of cAMP dependent protein kinase inhibitor H89 on meiotic resumption of pig oocytes with cumulus cells and a piece of parietal granulosa tissue (OCGs)

Addition of H89 (μM)	Duration of treatment (hr)	No. of oocytes examined*	No. (%) of GV oocytes	No. (%) of GVBD** oocytes					No. (%) of oocytes degenerating
				Total	D	MI	AI-TI	MII	
0	0	48	47 (98)^{a}	1 (2)^{d}	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)
50	2	35	30 (86)^{ac}	5 (14)^{bd}	3 (9)^{ab}	2 (6)^{bc}	0 (0)	0 (0)	0 (0)
	4	34	23 (68)^{ade}	9 (26)^{ab}	4 (12)^{ab}	5 (15)^{ac}	0 (0)	0 (0)	2 (6)^{e}
	6	32	22 (69)^{ade}	8 (25)^{abc}	2 (6)^{b}	3 (9)^{ac}	0 (0)	3 (9)	2 (6)^{de}
	24	43	40 (93)^{ab}	3 (7)^{cd}	2 (5)^{b}	1 (2)^{c}	0 (0)	0 (0)	0 (0)
75	2	34	17 (50)^{af}	8 (24)^{abc}	2 (6)^{b}	6 (17)^{ab}	0 (0)	0 (0)	9 (26)^{bd}
	4	36	14 (39)^{df}	10 (28)^{ab}	3 (8)^{ab}	7 (19)^{ab}	0 (0)	0 (0)	12 (33)^{abc}
	6	32	7 (22)^{f}	14 (44)^{a}	3 (9)^{ab}	9 (28)^{a}	1 (3)	1 (3)	11 (34)^{abc}
	24	44	34 (77)^{ad}	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	10 (23)^{cde}
100	2	33	15 (45)^{bcdf}	13 (39)^{ab}	4 (12)^{ab}	7 (21)^{ab}	2 (6)	0 (0)	5 (15)^{cde}
	4	34	13 (38)^{df}	12 (35)^{ab}	10 (29)^{a}	2 (6)^{bc}	0 (0)	0 (0)	9 (26)^{cd}
	6	32	12 (38)^{df}	6 (19)^{abc}	4 (13)^{ab}	2 (6)^{bc}	0 (0)	0 (0)	14 (44)^{abc}
	24	44	14 (32)^{ef}	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	30 (68)^{a}

*Oocyte-cumulus cell complexes with parietal granulosa tisuue were treated with H89 for different durations, and subsequently cultured in H89-free medium. After a total of 24 hr, oocytes were fixed and then examined. **GVBD: germinal vesicle breakdown, D: diakinesis, MI: the first metaphase, AI-TI: the first anaphase to the first telophase, and MII: the second metaphase. $^{a-f)}$ Values with different superscripts in the same column are significantly different ($P<0.05$, χ^2-test).

Table 2. Effect of cAMP dependent protein kinase inhibitor H89 on meiotic resumption of pig oocytes surrounded by cumulus cells (OCs)

Addition of H89 (μM)	Duration of treatment (hr)	No. of oocytes examined*	No. (%) of GV oocytes	No. (%) of GVBD** oocytes					No. (%) of oocytes degenerating
				Total	D	MI	AI-TI	MII	
0	0	55	52 (95)^{a}	3 (5)^{d}	1 (2)^{b}	0 (0)	0 (0)	2 (4)^{b}	0 (0)
50	2	49	40 (82)^{ab}	7 (14)^{cd}	3 (6)^{ab}	2 (4)^{e}	0 (0)	2 (4)^{b}	2 (4)^{dg}
	4	50	23 (46)^{bce}	25 (50)^{ab}	1 (2)^{b}	19 (38)^{ab}	3 (6)	2 (4)^{b}	2 (4)^{eg}
	6	51	14 (27)^{ef}	36 (71)^{a}	5 (10)^{ab}	29 (57)^{a}	1 (2)	1 (2)^{b}	1 (2)^{g}
	24	30	19 (63)^{acd}	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	11 (37)^{ab}
75	2	36	22 (61)^{acd}	14 (39)^{ab}	1 (3)^{b}	9 (25)^{acd}	1 (3)	3 (8)^{ab}	0 (0)
	4	36	6 (17)^{f}	25 (69)^{a}	4 (11)^{ab}	14 (39)^{ab}	3 (8)	4 (11)^{ab}	5 (14)^{bcdefg}
	6	33	15 (45)^{bdef}	13 (39)^{ab}	7 (21)^{a}	6 (18)^{bcde}	0 (0)	0 (0)	5 (15)^{bcdef}
	24	30	14 (47)^{acf}	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	16 (53)^{a}
100	2	34	13 (38)^{cf}	20 (59)^{ab}	1 (3)^{b}	11 (32)^{ac}	1 (3)	7 (21)^{a}	1 (3)^{fg}
	4	36	21 (58)^{ace}	9 (25)^{bc}	0 (0)	5 (14)^{bcde}	4 (11)	0 (0)	6 (17)^{bcdef}
	6	35	23 (66)^{acd}	3 (9)^{cd}	1 (3)^{b}	2 (6)^{de}	0 (0)	0 (0)	9 (26)^{ac}
	24	29	11 (38)^{bdef}	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	18 (62)^{a}

*Oocyte-cumulus cell complexes were treated with H89 for different durations, and subsequently cultured in H89-free medium. After a total of 24 hr, oocytes were fixed and then examined. **GVBD: germinal vesicle breakdown, D: diakinesis, MI: the first metaphase, AI-TI: the first anaphase to the first telophase, and MII: the second metaphase. $^{a-g)}$ Values with different superscripts in the same column are significantly different ($P<0.05$, χ^2 test).

Denuded oocytes were treated with 100 μM H89 for various durations in the culture medium supplemented with 6 mM hypoxanthine to prevent spontaneous meiotic maturation (Table 3). In the hypoxanthine-free medium, 75% of denuded oocytes underwent GVBD after 24 hr. Hypoxanthine significantly inhibited the spontaneous resumption of meiosis by 12%. In the hypoxanthine-supplemented medium, H89 increased the number of oocytes undergoing GVBD significantly. H89 treatment for 1/2 hr was most effective for oocyte GVBD.

Table 3. Effect of cAMP dependent protein kinase inhibitor H89 on meiotic resumption of denuded pig oocytes

Addition of hypoxanthine (mM)	Treatment of H89 (hr)	No. of oocytes examined*	No. (%) of GV oocytes	No. (%) of GVBD** oocytes					No. (%) of oocytes degenerating
				Total	D	MI	AI-TI	MII	
0	0	36	8 (22)^{b}	27 (75)^{a}	1 (3)	18 (50)^{a}	1 (3)	7 (19)	1 (3)^{c}
6	0	34	25 (74)^{a}	4 (12)^{c}	0 (0)	3 (9)^{b}	0 (0)	1 (3)	5 (15)^{bc}
6	1/6	36	24 (67)^{a}	10 (28)^{bc}	2 (6)	4 (11)^{b}	1 (3)	3 (8)	2 (6)^{c}
6	1/2	36	9 (25)^{b}	15 (42)^{ab}	2 (6)	11 (31)^{ab}	2 (6)	0 (0)	12 (33)^{ab}
6	1	35	3 (9)^{b}	11 (31)^{bc}	3 (9)	3 (9)^{b}	3 (9)	2 (6)	21 (60)^{a}
6	2	35	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	35 (100)

*Denuded pig oocytes were treated with 100 μM H89 for different durations with or without 6 mM hypoxanthine, and subsequently cultured in H89-free medium. After a total of 24 hr, oocytes were fixed and then examined. **GVBD: germinal vesicle breakdown, D: diakinesis, MI: the first metaphase, AI-TI: the first anaphase to the first telophase, and MII: the second metaphase. $^{a-c)}$ Values with different superscripts in the same column are significantly different ($P<0.05$, χ^2-test).

However, when the oocytes were treated with H89 over 1/2 hr, those undergoing GVBD decreased with the increment of degenerating oocytes.

Discussion

Meiotic resumption was induced by PKA inhibitor H89 in cumulus-enclosed pig oocytes as well as oocytes with cumulus and parietal granulosa tissue in the present study. Without gonadotropic stimulation, GVBD was scarcely induced in these oocytes under our culture conditions (see Table 1 and 2). We also examined the direct action of H89 on denuded pig oocytes, the meiotic resumption of which was inhibited by hypoxanthine. Hypoxanthine has been identified as the major inhibitory component of the low molecular weight of the follicular fluid responsible for inhibition of meiotic resumption of mouse oocytes [30]. It has been also reported that hypoxanthine similarly inhibits meiotic resumption of oocytes in other mammalian species such as bovine [8], monkey [31], rat [32] and pig [33]. Meiotic resumption of pig denuded oocytes was significantly inhibited by 6 mM hypoxanthine, although a significant number of denuded oocytes underwent GVBD after H89 treatment in the presence of hypoxanthine. These results suggest that PKA is involved in the meiotic arrest in pig oocytes similar to those of other species previously reported. Rose-Hellekant and Bavister showed the necessity of PKA for the maintenance of meiotic arrest in hamster and bovine oocytes in similar perturbation experiments of PKA using H89. Oocytes recovered from the ovaries of hamsters into which H89 was injected into the ovarian bursal cavity showed a significantly greater frequency of meiotic resumption [27]. They also reported that H89 interfered with forskolin- or IBMX-arrested bovine oocytes in a dose dependent manner [26].

GVBD was induced in about 70% of cumulus-enclosed oocytes by H89, but the maximal percentage of GVBD was 44% in the cumulus-enclosed oocytes with parietal granulosa tissue. It has been suggested that an inhibitor(s) of oocyte meiotic resumption is produced by granulosa cells, because the resumption is restricted when intact cumulus oocyte complexes are cultured in contact with parietal granulosa cells [34, 35]. Based on these reports, our results indicate that parietal granulosa tissue inhibits the meiotic resumption of the oocytes in a pathway other than PKA.

At high doses and long treatment, H89 showed a deleterious effect on pig oocytes without their meiotic resumption. The inhibition of PKA using another inhibitor H8, decreased the rate of GVBD of cumulus-enclosed pig oocytes in a dose dependent manner by approximately 12%, reaching a plateau at 100 μM [36]. Aside from meiotic arrest, active PKA, as the principle mediator of the key second messenger cAMP, affects multiple cellular events, including glycogen metabolism [37], gene transcription [38], regulation of cell shape and cytoskeletal dynamics [39], and control of cell growth and proliferation [40]. Long-term inhibition of PKA probably damages oocyte viability.

In *Xenopus* oocytes, Rime *et al.* [41, 42] suggested the possibility of PKA negatively regulating the activation of MPF (maturation promoting factor [43]). They proposed three possible regulations: 1) PKA promotes the phosphorylation of MPF catalytic subunit, $p34^{cdc2}$ by Wee 1/Mik 1 [44]; 2) PKA inhibits the dephosphorylation of $p34^{cdc2}$ by Cdc25 [45]; 3) PKA inhibits the association

of $p34^{cdc2}$ and its regulatory subunit cyclin B. MPF activation occurs after a few hours in *Xenopus* oocytes, while pig oocytes take about 30 hr for the activation [46]. Since meiotic arrest of oocytes are maintained by PKA in both species, it should be possible to identify the signal transduction cascade from PKA inhibition to MPF activation by using pig oocytes.

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