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Original article

Effects of protein kinase inhibitors on pig oocyte maturation *in vitro*

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Summary — Normal oocyte maturation depends on signal transmission between granulosa cells and the oocyte. We have analysed the effects of inhibiting (I) cyclic AMP-dependent protein kinase (protein kinase A, PK-A), (II) Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C, PK-C) and (III) calmodulin (CaM) on pig oocyte maturation *in vitro*, protein synthesis and phosphorylation. The inhibition of PK-A using a specific inhibitor H8, decreased the maturation rate (rate of germinal vesicle breakdown, GVBD) of cumulus-enclosed pig oocytes in a dose-dependent manner by ≈ 12%, reaching a plateau at 100 μM. The inhibition of PK-C with H7, an inhibitor with some side-effects on PK-A, decreased the maturation rate of cumulus-enclosed oocytes in a dose-dependent manner to a maximum of 20% at a concentration of 100 μM. The calmodulin antagonist W7 up to a concentration of 200 μM had no effects on maturation of cumulus-enclosed pig oocytes. None of the inhibitors (H7, H8 and W7) altered the patterns of protein synthesis of either pig oocytes and cumulus cells after maturation *in vitro*. Oocyte phosphoprotein patterns were, however, clearly changed by W7. Cumulus cell protein phosphorylation patterns were changed by all 3 agents. Since inhibition of cyclic AMP and Ca^{2+} phospholipid pathways by PK-A and PK-C blocking chemicals affected only a limited proportion of oocytes (12 and 20%, respectively) and inhibition of Ca^{2+} binding to CaM was without effect on oocyte maturation, we conclude that these pathways modulate rather than regulate oocyte maturation in the pig.

pig / oocyte maturation / protein kinase C / cyclic AMP-dependent protein kinase / calmodulin

Résumé — Effets des inhibiteurs des protéines kinases sur la maturation des ovocytes de porc *in vitro*. *La maturation normale de l'ovocyte dépend de la transmission de signaux entre les cellules de la granulosa et l'ovocyte. Nous avons étudié l'effet d'une inhibition*
– de la protéine kinase AMP_c-dépendante (protéine kinase A, PK-A),
– de la protéine kinase Ca^{2+}/phospholipide-dépendante (protéine kinase C, PK-C), et
– de la calmoduline (CaM), sur la maturation, la synthèse protéique et la phosphorylation d'ovocytes de porc in vitro.
L'inhibition de la PK-A, par l'inhibiteur spécifique H8, diminuait le taux de maturation (taux d'éclatement de la vésicule germinale) des ovocytes entourés du cumulus avec une intensité proportionnelle à la dose, atteignant un maximum de 12% à une concentration en H8 de 100 μM. L'inhibition de la PK-C par H7, composé qui inhibe aussi partiellement la PK-A, diminuait le taux de maturation des ovocytes entourés du cumulus proportionnellement à la dose avec un maximum de 20% à une concentration en H7 de 100 μM. L'inhibiteur W7 de la calmoduline, jusqu'à une concentration de 200 μM, n'avait pas d'effet sur la maturation des ovocytes entourés de cumulus. Aucun des inhibiteurs utilisés (H7, H8 et

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W7) n'a modifié qualitativement la synthèse protéique des ovocytes ou des cellules du cumulus après maturation in vitro. *La répartition des phosphoprotéines était cependant clairement modifiée par W7 dans les ovocytes et par les 3 agents chimiques dans les cellules du cumulus. Puisque l'inhibition des voies de l'AMP_c et du Ca^{2+}/phospholipide par les substances bloquant l'activité de la PK-A ou la PK-C affectait seulement une proportion limitée des ovocytes (respectivement 12 et 20%), et que l'inhibition de la fixation du Ca^{2+} à la calmoduline était sans effet sur la maturation, nous concluons que ces voies modulent plutôt que régulent la maturation des ovocytes de porc* in vitro.

porc / maturation des ovocytes / protéine kinase C / protéine kinase AMP_c dépendante / calmoduline

INTRODUCTION

Oocytes are coupled to the surrounding granulosa cells throughout oogenesis by gap junctions. After growing to the full size, oocytes resume maturation after receiving the appropriate hormonal stimulus. The period of meiotic arrest during the growth phase and the subsequent resumption of meiosis during maturation appear to be mediated by factors from the cumulus cells which are transferred into the oocytes *via* cellular junctions. There are a substantial number of reports supporting the hypothesis that cyclic adenosine monophosphate (cyclic AMP) is involved in maintaining meiotic arrest in mouse, rat and amphibian oocytes (mouse: Cho *et al,* 1974; Bornslaeger *et al,* 1986a; rat: Magnusson and Hillensjö, 1977; Dekel and Beers, 1978; amphibia: reviewed by Maller, 1983). This is based on the finding that cyclic AMP derivatives, such as dibutyryl cyclic AMP (dbcAMP), and activators of adenylate cyclase, like forskolin and cholera toxin, or inhibitors of phosphodiesterase, like 3-isobutyl-1-methylxanthine, inhibit germinal vesicle breakdown (GVBD). The cyclic AMP-dependent protein kinase (PK-A) is involved in the inhibitory action of cyclic AMP (Bornslaeger *et al,* 1986a). The dbcAMP-induced meiotic arrest can be overcome by increasing intracellular Ca^{2+} levels by Ca^{2+}-ionophore treatment (Paleos and Powers, 1981; Powers and Paleos, 1982). Metabolism and action of calcium ions in eukaryotic cells are regulated by the interaction with calmodulin (CaM). CaM antagonists inhibit oocyte maturation in the mouse (Bornslaeger *et al,* 1986b; Sato and Koite, 1987). Furthermore, oocyte maturation is inhibited by activators of the Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C, PK-C) (Urner and Schorderet-Slatkine, 1984; Bornslaeger *et al,* 1986b). From these reports it is obvious that at least 2 intracellular second messenger systems are involved in the onset of oocyte maturation. Intracellular mediators of these second messenger systems are protein kinases whose activities are in turn regulated by them.

Little is known about the importance of the above-mentioned second messengers during oocyte maturation in domestic species. Pig oocytes are able to synthesize their own cyclic AMP (Racowsky, 1985). However, inhibitory effects of dbcAMP on pig oocyte maturation have been described (Rice and McGauchey, 1981).

To learn more about the importance of different second messenger systems and their mediating protein kinases during pig oocyte maturation, we blocked the activity of PK-A and PK-C with inhibitors and prevented binding of Ca^{2+} to CaM with an antagonist. Naphthalene sulfonamides, *eg* W7 (*N*-[6-aminohexyl]-5-chloro-1-naphthalene sulfonamide), are cell-permeable CaM antagonists (Hidaka *et al,* 1978). When the naphthalene ring of these sulfonamides is replaced by isoquinoline the resulting

agents are no longer CaM antagonists, but rather compounds that directly suppress protein kinase activity. The isoquinoline sulfonamides H7 (1-[5-isoquinolinesulfonyl]-2-methylpiperazine) and H8 (*N*-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide) are specific PK-C and PK-A inhibitors (Hidaka *et al*, 1984). In the present study we inhibited PK-A, PK-C or CaM in cumulus-enclosed pig oocytes and had them analyzed for their potential to undergo GVBD and synthesize and phosphorylate proteins.

MATERIALS AND METHODS

Collection and culture of oocytes

Pig ovaries were collected from a local slaughterhouse and transported to the laboratory in a thermo-container within 30 min. To maximize oocyte numbers without decreasing oocyte quality, whole ovaries were dissected with a multi-razor blade dissection system. After washing, cumulus-enclosed oocytes with a minimum diameter of 115 μm were selected. For experiments with denuded oocytes selected cumulus-enclosed oocytes were mechanically denuded. Dissection, washing and selection was carried out in medium 199 supplemented with 5% fetal calf serum. After selection oocytes were matured *in vitro* in medium 199 supplemented with 10% fetal calf serum, 1 mg/ml glutamine, 0.7 mg/ml L-ascorbic acid, 0.35 mg/ml insulin, 0.3 IU/ml hFSH, and 0.3 IU/ml hLH using a gentle agitation system. The activities of PK-A, PK-C or CaM were inhibited by the addition of H7, H8 or W7 to final concentrations of 0, 50, 100, 150 or 200 μM.

Radiolabelling of oocyte proteins

In order to study protein synthesis, cumulus enclosed pig oocytes were incubated for 2 h in [^{35}S]-methionine (Tran ^{35}S-label, spec act > 37 TBq/mmol, ICN) at an isotope concentration of 37 MBq/ml using phosphate-free Hepes-buffered M2 medium. Protein phosphorylation was studied by labelling oocytes for 2 h in [^{32}P]-orthophosphate (carrier-free, ICN) at an isotope concentration of 37 MBq/ml using phosphate-free Hepes-buffered M2 medium. All labelled oocytes were denuded of cumulus cells before collection for electrophoresis. Labelling of the inhibitor treated oocytes was performed in the presence of 100 μM H8, H7 or W7, respectively.

Assessment of oocyte maturation

The effects of the inhibitors of PK-A, PK-C or CaM on *in vitro* maturation of cumulus-enclosed or denuded pig oocytes were determined by fixing whole oocytes in ethanol–acetic acid (3:1) and staining with orcein. All statistical comparisons were made by χ^2-square tests.

Gel electrophoresis

Samples were collected in 2 μl phosphate-buffered saline and lysed with 15 μl of 2x-concentrated SDS–sample buffer and 2 μl were removed for measuring the incorporation of label into TCA-precipitable material. One-dimensional electrophoresis was performed on 8–15% linear gradient SDS–polyacrylamide gels (Laemmli, 1969). Direct autoradiography was carried out on dried gels using Hyperfilm β-max (Amersham).

RESULTS

Pig oocytes dissected from ovaries and cultured *in vitro* undergo germinal vesicle breakdown after ≈ 24 h. The metaphase I stage than persists for ≈ 8 h. The timing of the maturation cycle of individual oocytes may, however, vary by ≈ 3–4 h from the median outlined above.

Effects of the protein kinase inhibitor H8 on GVBD

Cumulus-enclosed pig oocytes were matured *in vitro* for 30 h in the presence of increasing concentrations of H8 (table I).

Table I. Effects of H8 on cumulus enclosed pig oocytes after 30 h *in vitro*.

	H8 concentrations (μM)									
	0		*50*		*100*[a]		*150*[a]		*200*[a]	
Germinal vesicle stage	44	(22.9%)	43	(29.5%)	72[b]	(36.7%)	52[b]	(32.7%)	56[b]	(35.0%)
Metaphase I	139	(72.4%)	102	(69.9%)	118[c]	(60.2%)	102	(64.2%)	102	(63.8%)
Anaphase I/ telophase I	8	(4.2%)	1	(0.7%)	4	(2.0%)	4	(2.5%)	2	(1.3%)
Metaphase II	1	(0.5%)	0		2	(1.0%)	1	(0.6%)	0	
No of oocytes	192		146		196		159		160	

[a] The distributions of maturation stages after treatment with 100, 150, or 200 μM H8 show a statistically significant difference from 0 μM H8 with probabilities of $P \leq 0.001$, or $P \leq 0.01$, or $P \leq 0.001$, respectively. [b] The number of oocytes unable to mature (germinal vesicle stage) after treatment with 100, 150 or 200 μM H8 are statistically significantly higher compared to 0 μM H8 with probabilities of $P \leq 0.001$, $P \leq 0.005$, or $P \leq 0.001$, respectively. [c] The number of oocytes reaching metaphase I stage after treatment with 100 μM H8 is statistically significantly lower compared to 0 μM H8 with a probability of $P \leq 0.01$.

Treatment of pig oocytes with H8 decreased the proportion of oocytes which induced GVBD in a dose-dependent manner from 0 to 100 μM, reaching a plateau with no further increase when higher concentrations were used. The plateau was ≈ 12% below control levels. The inhibitory effect of H8 was statistically significantly different between control and 100, 150, or 200 μM treatment groups (table I). There was no difference between the control and the 50 μ-M treatment group and between the individual treatments ($P > 0.05$).

Similar experiments were performed with denuded pig oocytes (data not shown). High concentrations of H8 (100 μM or over) did not affect oocyte maturation *in vitro*. However, there was a slight improvement of maturation in denuded pig oocytes with 50 μM H8.

Effects of the protein kinase inhibitor H7 on GVBD

H7 had a similar dose-dependent but a more pronounced inhibitory effect on the maturation of cumulus-enclosed pig oocytes after 30 h culture *in vitro* than was observed after H8 treatment (table II). Increasing concentrations of H7 decreased the proportion of oocytes able to mature in a dose-dependent manner from 0 to 100 μM, reaching a plateau with no further increase when higher concentrations of the inhibitor were used. This plateau was ≈ 17–24% below control levels. The inhibitory effect of H7 was statistically significantly different between control oocytes and H7-treated oocytes (100, 150, 200 μM, table II). There was no difference between the control and the 50 μM group and between different treatments ($P > 0.05$).

In contrast to H8 however, H7 had a significant effect on the *in vitro* maturation of denuded pig oocytes (data not shown). The effect was similar to that on cumulus-enclosed oocytes, with a plateau at 100 μM or at higher concentrations of H7.

Effects of a combined treatment of H7 and H8 on GVBD

Pig oocytes were treated with 50 μM H7 and 50 μM H8 and the results compared

Table II. Effects of H7 on cumulus enclosed pig oocytes after 30 h *in vitro*.

	H7 concentrations (μM)				
	0	*50*	*100*[a]	*150*[a]	*200*[a]
Germinal vesicle stage	46 (26.1%)	34 (33.3%)	42[b] (43.3%)	38b (44.7%)	49[b] (50.0%)
Metaphase I	123 (69.8%)	65 (63.7%)	51[c] (52.6%)	46 (54.1%)	48[c] (49.0%)
Anaphase I/telophase I	6 (3.4%)	3 (2.9%)	4 (4.1%)	1 (1.2%)	1 (1.0%)
Metaphase II	1 (0.6%)	0	0	0	0
No of oocytes	176	102	97	85	98

[a] The distributions of maturation stages after treatment with 100, 150, or 200 μM H8 show a statistically significant difference from 0 μM H8 with probabilities of $P \leq 0.005$, or $P \leq 0.00\,05$, or $P \leq 0.001$, respectively. [b] The number of oocytes unable to mature (germinal vesicle stage) after treatment with 100, 150 or 200 μM H7 are statistically significantly higher compared to 0 μM H7 with probabilities of $P \leq 0.001$, $P \leq 0.001$, or $P \leq 0.001$, respectively. [c] The number of oocytes reaching metaphase I stage after treatment with 100 or 200 μM H7 is statistically significantly lower compared to 0 μM H7 with probabilities of $P \leq 0.01$ or $P \leq 0.01$ respectively.

with the expected distribution calculated as a simple additive effect of the single treatments with 50 μM H7 and 50 μM H8, respectively. The combined treatment of cumulus enclosed pig oocytes reduced the proportion of oocytes able to mature *in vitro* (table III). However, the observed distribution of maturation stages was not different from that expected, indicating simple additive effects between the treatments.

Table III. Effects of a combined treatment of H7 and H8 on cumulus enclosed pig oocytes after 30 h *in vitro*.

	H7 or H8 concentration (μM)		*Expected distribution*[e]
	0[a]	*50 H7 + 50 H8*[b]	
Germinal vesicle stage	90 (24.5%)	52[c] (41.6%)	48 (38.2%)
Metaphase I	262 (71.2%)	69[d] (55.2%)	77[f] (61.8%)
Anaphase I/ telophase I	14 (3.8%)	1 (0.8%)	
Metaphase II	2 (0.5%)	3 (2.4%)	
No	368	125	125

[a] Sum of oocytes from groups 0 μM H7 (table II) and 0 μM H8 (table I). [b] The distribution of maturation stages after a combined treatment of 50 μM H7 and 50 μM H8 shows a statistically significant difference from control oocytes (0 μM) with a probability of $P \leq 0.001$. [c] The number of oocytes unable to mature (germinal vesicle stage) after a combined treatment is statistically significantly higher compared to control oocytes with a probability of $P \leq 0.001$. [d] The number of oocytes reaching metaphase I after a combined treatment is statistically significantly lower compared to control oocytes with a probability of $P \leq 0.01$. [e] The expected numbers of oocytes for the different maturation stages were calculated as an additive effect from the numbers given for the single treatments with 50 μm H8 and 50 μm H7 in tables I and II, respectively. There is no difference between observed and expected distribution. [f] The number given stands for the sum of the maturation stages metaphase I, anaphase I/ telophase I and metaphase II.

Effects of the calmodulin antagonist W7 on GVBD

Cumulus enclosed (table IV) and denuded (data not shown) pig oocytes treated with increasing concentrations of W7 (50, 100, 150 and 200 μM) matured *in vitro* at rates comparable to control oocytes. There were no significant differences between groups.

Protein synthesis and phosphorylation pattern of pig oocytes and cumulus cells after inhibitor treatment

Cumulus-enclosed pig oocytes were matured *in vitro* in the presence of the protein kinase inhibitors H7 or H8 or the CaM antagonist W7. During the last 2 h of *in vitro* culture, the oocytes were labelled with [^{35}S]-methionine or [^{32}P]-orthophosphate to analyze protein synthesis or protein phosphorylation, respectively. After labelling, the oocytes were mechanically denuded and oocytes and cumulus cells were collected separately and analyzed by 1-dimensional electrophoresis and autoradiography.

After treatment of cumulus-enclosed oocytes with H7, H8 and W7 the levels of protein synthesis and phosphorylation in oocytes and cumulus cells were comparable with the controls, respectively (data not shown). The protein synthesis pattern of pig oocytes and cumulus cells after treatment with the inhibitors were indistinguishable from those of metaphase I stage oocytes and the control cumulus cells, respectively (fig 1).

Protein phosphorylation pattern of oocytes were not altered after H7 and H8 treatment. W7, however, inhibited the phosphorylation of a 200-kDa protein in oocytes (fig 2). Cumulus cell phosphorylation was affected by all 3 inhibitors. H8 increased the phosphorylation of a 23-kDa protein. H7 decreased phosphorylation of 33 and 28 kDa bands. Phosphorylation of the 28 kDa protein was also inhibited by W7 (fig 2).

DISCUSSION

We report on the effects of isoquinoline sulfonamides H7 and H8 as inhibitors of cyclic AMP-dependent protein kinase (PK-A) and Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C, PK-C) and of the naphthalene sulfonamide W7 as an antagonist of calmodulin (CaM) on pig oocyte maturation *in vitro*. Our work was based on the hypothesis that by using these cell-permeable substances we would be able to discriminate between the roles which

Table IV. Effects of W7 on cumulus enclosed pig oocytes after 30 h *in vitro*.

	W7 concentration (μM)				
	0	*50*	*100*	*150*	*200*
Germinal vesicle stage	22 (22.4%)	26 (27.1%)	25 (29.1%)	26 (28.0%)	18 (20.7%)
Metaphase I	69 (70.4%)	66 (68.8%)	57 (66.3%)	61 (65.6%)	69 (79.3%)
Anaphase I/telophase I	5 (5.1%)	4 (4.2%)	1 (1.2%)	2 (2.2%)	0
Metaphase II	2 (2.0%)	0	3 (3.5%)	4 (4.3%)	0
No of oocytes	98	96	86	93	87

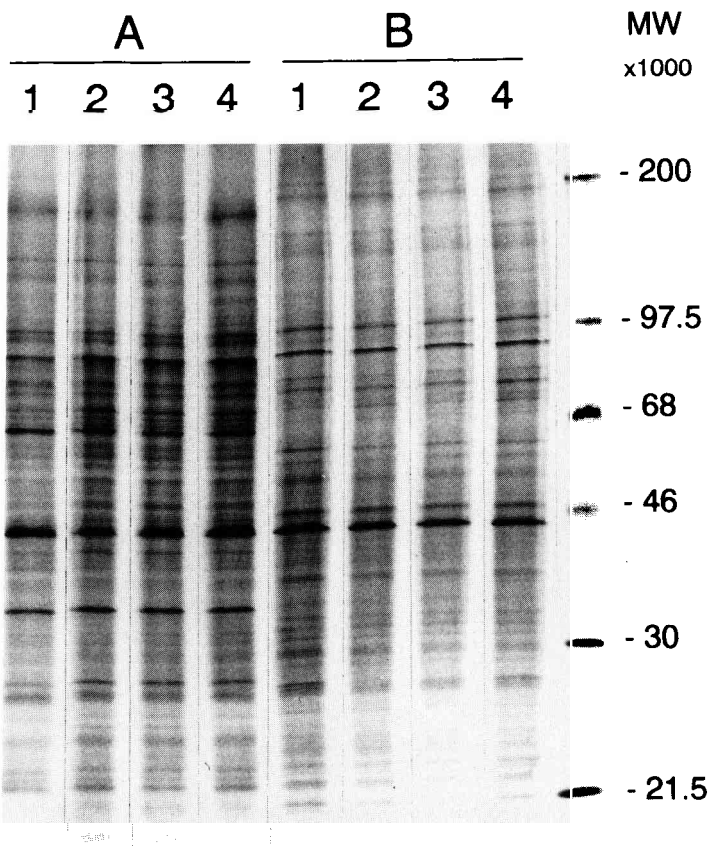

Fig 1. Autoradiogram of [^{35}S]-methionine labelled proteins synthesized by pig oocytes (A) and cumulus cells (B) cultured for 30 h in the presence of: 1) no inhibitors; 2) 100 μM H7; 3) 100 μM H8; or 4) 100 μM W7. Proteins were separated by SDS gradient polyacrylamide gel electrophoresis.

PK-A, PK-C and CaM mediated processes would play during oocyte maturation. The data presented indicate that PK-A, PK-C and/or CaM have modulating rather than dominating functions and that most of the effects are mediated through cumulus cells. In a dose-dependent manner inhibition of PK-A and PK-C with H8 and H7, respectively, slightly decreased the percentage of cumulus-enclosed oocytes able to break down their nuclear membrane (GVBD); from 76% in controls to ≈ 64% or 54% after H8 and H7 treatment, respectively. Even at very high concentrations (> 100 μM), H8 had no effect on the maturation of denuded oocytes. However, inhibition of protein kinases with H7 decreased the rate of GVBD in denuded pig oocytes in a dose-dependent manner. There were no obvious changes in either protein synthesis or phosphorylation after treatment of oocytes with H8 or H7, indicating that the phosphorylation of only a small undectable proportion of oocyte proteins could be de-

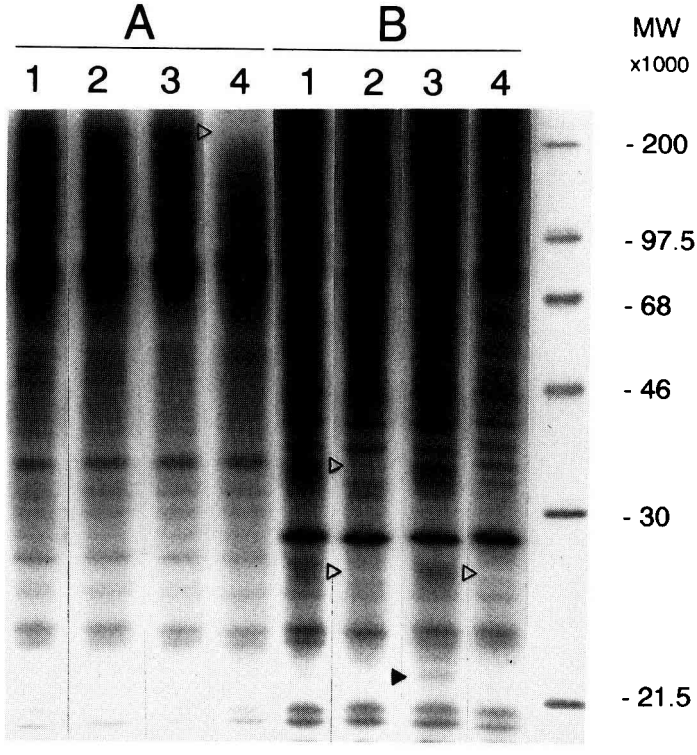

Fig 2. Autoradiogram of [^{32}P]-orthophosphate labelled proteins of pig oocytes (A) and cumulus cells (B) cultured for 30 h in the presence of: 1) no inhibitors; 2) 100 μM H7; 3) 100 μM H8; or 4) 100 μM W7. Proteins were separated by SDS gradient polyacrylamide gel electrophoresis. Open arrowheads indicate a decrease and filled arrowheads an increase compared to control groups.

pendent on PK-A or PK-C activity. By contrast, cumulus cell phosphorylation was markedly and selectively altered by each of the protein kinase inhibitors. Thus, H7 decreased phosphorylation of 33- and 28-kDa proteins whilst H8 increased phosphorylation in a 23-kDa protein. We interpret these results as suggesting that these agents exert a direct effect on cumulus cells, which is then reflected by an indirect effect on oocyte maturation.

The CaM antagonist W7 had no effect on pig oocyte maturation *in vitro* and on protein synthesis of oocytes and cumulus cells. However, phosphorylation of a 200-kDa protein in oocytes was inhibited and that of a 28-kDa protein in cumulus cells was decreased.

Naphthalene sulfonamides such as W7 are potent CaM antagonists with a K_i of ≈ 40 μM to inhibit Ca^{2+}–CaM dependent enzymatic reactions *in vitro* and *in vivo* (Hidaka *et al,* 1978). W7 blocks GVBD in mouse oocytes completely at concentrations > 30 μM (Bornslaeger *et al,* 1984). At 10 μM W7 inhibits cleavage of 1-cell

mouse embryos by inhibiting DNA replication (Poueymirou and Schultz, 1990). Under these conditions protein synthesis is unaffected and the activation of the embryonic genome occurs at the correct time. If W7 treatment is started after completion of S-phase, it is no longer able to prevent subsequent cleavage. Therefore it seems that in the mouse the CaM antagonist W7 inhibits oocyte maturation at the G2- to M-phase border and the first cleavage at the G1- to S-phase transition. In murine (Kaplan *et al,* 1982; Bornslaeger *et al,* 1984) and *Xenopus* oocytes (Cartaud *et al,* 1980; Wasserman and Smith, 1981) as in somatic tissues (Means *et al,* 1982), CaM constitutes ≈ 0.3% of total cellular proteins. Possible targets for Ca^{2+}–CaM action during mouse and *Xenopus* oocyte maturation are Ca^{2+}–CaM modulated phosphodiesterases and protein phosphatases. However, Ca^{2+}–CaM modulated phosphodiesterases account for < 5% of the cyclic AMP hydrolysis in oocytes (Allende and Allende, 1982; Bornslager *et al,* 1984). Protein phosphatases play an important role during mouse and *Xenopus* oocyte maturation (Huchon *et al,* 1981; Foulkes and Maller, 1982; Rime *et al,* 1990). In contrast to the findings in the mouse, W7 at concentrations up to 200 μM had no effect on pig oocyte maturation *in vitro* (table IV). Ca^{2+}–CaM mediated processes do not seem to play an important regulatory role during onset of oocyte maturation in the pig. Despite the use of high concentrations of the CaM antagonist W7, we were unable to detect any changes in the rate of GVBD and protein synthesis in oocytes. However, W7 altered the protein phosphorylation pattern in oocytes as well as in cumulus cells (fig 2). The importance of phosphorylation of the 200-kDa protein in oocytes, which is inhibited by W7, for further maturation and for oocyte activation and of the 28-kDa protein in cumulus cells for cumulus cell function remains to be established.

The isoquinoline sulfonamides H7 and H8 are derivatives of naphthalene sulfonamides but have the naphthalene ring replaced by isoquinoline (Hidaka *et al,* 1984, 1991). On basis of their different inhibitory constants (K_i) for PK-A and PK-C, it is possible to differentiate between their effects on each of the 2 protein kinases. However, although they have different affinities, these agents affect both PK-A and PK-C as well as other protein kinases. H7 and H8 bind to the ATP-binding site of the affected protein kinases (Hidaka *et al,* 1984). PK-A and PK-C are inhibited by H7 or H8 with K_i's of 3.0 and 1.2 or with K_i's of 6.0 and 15 μM, respectively (Hidaka *et al,* 1984). Since the K_i's of H7 and H8 towards PK-A differ by a factor of ≈ 2 and towards PK-C by a factor of ≈ 10 respectively, we could expect that PK-A would be inhibited by both agents (with H8 being slightly more powerful) and that PK-C would be inhibited almost exclusively by H7 in the range of concentrations used in our experiments.

Rat oocyte maturation induced by GnRH or LH in intact follicles *in vitro* is inhibited by H7 and H8 with IC_{50}'s of 20 and 80 or 20 and 100 μM, respectively (Dekel *et al,* 1990). Inhibition of pig oocyte maturation with H7 and H8 differs from the rat in the fact that in cumulus-enclosed oocytes, both agents decrease maturation rate in a dose-dependent manner up to a concentration of 100 μM. Higher concentrations of H7 and H8 had no further inhibitory effects (tables I and II). Like the rat, H7 is the more powerful inhibitor of oocyte maturation in the pig. The effective concentrations of H8 in the rat and pig experiments are in a comparable range. However, the rat seems to be more sensitive to H7 than the pig. The relative potencies of the 2 agents in inhibiting GnRH- or LH-induced oocyte maturation in the rat are proportional to the K_i's of the inhibitors toward PK-C rather than toward PK-A (Dekel *et al,* 1990). A somehow similar dynamic

was seen in our experiments with cumulus-enclosed pig oocytes.

The results of the experiments with H8 seem to indicate that the normal activity of PK-A in oocytes plays only a minor role during oocyte maturation and that the kinase activity in the cumulus cells is the main modulator for the observed effects on oocyte maturation. In accordance with this is the finding that the only H8 induced changes in protein phosphorylation were seen in cumulus cells. The phosphorylation of a 23-kDa protein was increased. This paradoxical phenomenon, *ie* that a protein kinase inhibitor increases protein phosphorylation, may be explained by the fact that for example the phosphoprotein phosphatase 1 is activated by PK-A (Ingrebritsen and Cohen, 1983; Hemmings Jr *et al,* 1984).

It must however be recalled that the maturation of only a small proportion of cumulus-enclosed pig oocytes (15–20%) is inhibited by blocking PK-A activity. Surprisingly, activators of PK-A activity like dbcAMP when used at high concentrations (280–1 000 μM), decrease maturation of cumulus-enclosed oocytes to the same limited degree as seen after inhibition of PK-A activity (Rice and mcGaughy, 1981; Racowsky, 1983; Petr *et al,* 1991). The response in denuded oocytes is, however, different – with activators of PK-A decreasing maturation (Rice and McGaughey, 1981) whilst inhibitors of PK-A activity are without effect.

Cyclic AMP regulatory processes play a unique dominating role in the onset of mouse oocyte maturation (Cho *et al,* 1974; Wassarman *et al,* 1976; Bornslaeger *et al,* 1986a, b). High cyclic AMP levels prevent maturation and spontaneous maturation *in vivo* and *in vitro* is paralleled by a decline of cyclic AMP levels in the oocyte (Schultz *et al,* 1983; Vivarelli *et al,* 1983). Bornslaeger *et al* (1986a) were able to show that PK-A is directly involved in the cyclic AMP-dependent meiotic arrest in mouse oocytes. In contrast to the mouse, there is no parallel decline in cyclic AMP levels during maturation in pig oocytes (Racowsky, 1985). All these findings in the pig suggest a more modulatory role of cyclic AMP and of PK-A activity during oocyte maturation compared with their more dominating role in the mouse.

H7 compared to H8 is the more powerful protein kinase inhibitor in blocking GVBD in the rat (Dekel *et al,* 1990) as well as in the pig (tables I and II). The relative potencies of the 2 agents correspond to their K_i's towards PK-C rather than to PK-A. From experiments in the rat we know that LH is able to stimulate cyclic AMP and inositol triphosphate in granulosa cells (Davies *et al,* 1986), and therefore most probably PK-A and PK-C. H7 inhibited the phosphorylation of 32 and 28-kDa proteins in pig cumulus cells. These changes in cumulus cell protein phosphorylation are different from those induced by H8 (increased phosphorylation are different from those induced by H8 (increased phosphorylation at 23 kDa; fig 3) and are therefore most probably PK-C-mediated. The fact that H7 and W7 induced a similar inhibition of phosphorylation at 28 kDa in cumulus cells indicates a role of Ca^{2+}-mediated processes in cumulus cell action on pig oocyte maturation. Activators of PK-C, like phorbol esters, inhibit maturation of denuded mouse oocytes and of follicle-enclosed rat oocytes (Urner and Schorderet-Slatkine, 1984; Aberdam and Dekel, 1985; Bornslaeger *et al,* 1986b; Alexandre and Mulnard, 1988). In amphibians progesterone- and phorbol ester-induced oocyte maturation is inhibited by H7 and W7 (Kwon and Lee, 1991). A > 50% inhibition of maturation is achieved with 100 μM H7 or W7 for both activators.

Beside the effects on cumulus cells, H7 exerted direct effects on pig oocytes. However, after inhibition of PK-C with H7 a

large proportion of pig oocytes escaped the inhibitory action. Again like the situation of the cyclic AMP-dependent system, the PK-C action during pig oocyte maturation is a more modulatory system compared to more dominant role played by PK-C during oocyte maturation in mouse, rat and amphibians. The results from our experiments in which pig oocytes were treated with both inhibitors (table III) suggest that there are no obvious more-than-additive interactions between PK-A and PK-C activities in cumulus-enclosed pig oocytes. The observed results are not different from the expected effects, assuming simple additive interactions.

The puromycin analog 6-dimethylaminopurine (6-DMAP) totally inhibits meiotic maturation of marine invertebrate and mammalian oocytes (Neant and Guerrier, 1988a,b; Neant *et al,* 1989; Rime *et al,* 1989; Rime and Ozon, 1990; Motlik and Rimkevicova, 1990; Fulka *et al,* 1991; Szöllösi *et al,* 1991) by inhibiting protein phosphorylation. Protein synthesis is not a affected by 6-DMAP (Neant and Guerrier, 1988b; Rime *et al,* 1989). The target kinases for 6-DMAP are cyclic AMP and Ca^{2+}-independent serine and threonine kinases Neant and Guerrier, 1988b). Therefore, 6-DMAP seems to act on protein kinases not directly involved in the interaction between cumulus cells and oocyte and the known second messenger systems.

We conclude from the combined result of earlier reports and our present results that the pathways involving PK-A, PK-C and CaM play more minor and more modulatory role in pig oocytes as compared to the more dominating roles in mouse, rat and amphibians. An interaction between cumulus cells and oocyte in transferring second messengers is obvious. Cumulus cells as well as oocytes could be direct or indirect targets of the inhibitors. It remains to be established which second messenger molecules are generated in pig cumulus cells in response to the systemic hormonal stimuli which are then transferred to the oocyte to release meiotic arrest and initiate maturation and which oocyte factors feed back to regulate cumulus cell function. However, a major limitation of all *in vitro* oocyte maturation systems in assessing the interaction between cumulus cells and oocyte in the regulation of oocyte maturation is the fact that oocytes start to mature spontaneously after release from their follicles. New improved culture systems should be developed in which oocytes remain arrested in the GV stage *in vitro* until they are released by hormonal stimuli.

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