

Evidence that growth factors IGF-I, IGF-II and EGF can stimulate nuclear maturation of porcine oocytes via intracellular protein kinase A

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Abstract — The aim of our in vitro experiments was to study the role of growth factors and protein kinase A (PKA)-dependent intracellular mechanisms in the control of nuclear maturation of porcine oocytes. Oocytes were cultured with or without growth factors (IGF-I, IGF-II, EGF; 10 ng·mL⁻¹ medium) and inhibitors of PKA (Rp-cAMPS or KT5720; 100 ng·mL⁻¹). Stages of meiosis were determined from the structure of chromosomes after staining with Giemza. Intracellular levels of PKA were evaluated immunocytochemically using primary antisera against the PKA regulatory and catalytic subunits and by Western immunoblotting using primary antiserum against the PKA catalytic subunit. It was found that after 24 h culture the majority of oocytes had resumed nuclear maturation (they were at a stage of meiosis after diplotene) and that after 48 h culture the majority of cells had completed maturation (they had reached metaphase II of meiosis). Addition of IGF-I, IGF-II or EGF, or a combination of IGF-I and EGF, significantly increased the proportion of oocytes which resumed and completed meiosis. Immunocytochemistry demonstrated a significant increase in the proportion of cells containing catalytic and, in some cases, the regulatory subunits of PKA after addition of IGF-I, IGF-II and EGF. Immunoblotting showed the presence of 2 forms of the PKA catalytic subunit within the oocytes (MW approximately 52 and 40 kD). EGF, but not IGF-I or IGF-II, increased the content of both isoforms. Inhibitors of PKA, when given alone, did not substantially influence the proportion of oocytes which resumed or completed meiosis. However, Rp-cAMPS and KT5720 both prevented the stimulatory effects of IGF-I, IGF-II and EGF on the resumption and completion of oocyte maturation. The present observations suggest (1) that IGF-I, IGF-II and EGF are potent stimulators of both resumption and completion of porcine oocyte nuclear maturation, (2) that PKA is present in oocytes, and (3) that PKA-dependent intracellular mechanisms can mediate the action of growth factors on porcine oocytes.

insulin-like growth factor / epidermal growth factor / protein kinase A / meiosis

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Résumé — Les facteurs de croissance IGF-I, IGF-II et EGF peuvent stimuler la maturation nucléaire de l'ovocyte de porc par la voie intracellulaire de la protéine kinase A. Ces expériences ont pour but d'étudier in vitro le rôle des facteurs de croissance et des mécanismes intracellulaires dépendant de la protéine kinase A (PKA) vis-à-vis du contrôle nucléaire des ovocytes porcins. Des ovocytes ont été cultivés avec ou sans facteurs de croissance (IGF-I, IGF-II, EGF ; $10 \text{ ng}\cdot\text{mL}^{-1}$ de milieu) et avec ou sans des inhibiteurs de la PKA (Rp-cAMPS, KT5720 ; $100 \text{ ng}\cdot\text{mL}^{-1}$). Les stades de la méiose ont été déterminés par examen des chromosomes après coloration par le Giemza. Les niveaux intracellulaires de PKA ont été évalués par immunocytochimie en utilisant un anticorps primaire de la sous-unité régulatrice et de la sous-unité catalytique de PKA et par Western immunoblot avec des anticorps primaires dirigé contre la sous-unité catalytique. Après 24 h de culture, la majorité des ovocytes a repris sa maturation nucléaire (stade diplotène dépassé) et elle l'a terminée (stade métaphase II atteint) après 48 h. L'addition de IGF-I, IGF-II ou d'EGF, ou une combinaison de IGF-I et d'EGF, accroît significativement la proportion d'ovocytes qui ont repris et terminé leur méiose. L'addition de IGF-I, IGF-II ou d'EGF s'accompagne d'un accroissement significatif des cellules exprimant la sous-unité catalytique et dans certains cas la sous-unité régulatrice de la PKA (immunocytochimie). La présence de deux formes de la sous-unité catalytique à l'intérieur des ovocytes est montrée en immunoblot (PM 52 et 40 kD environ). EGF mais non IGF-I ou IGF-II augmente le contenu des deux isoformes. Les inhibiteurs de la PKA, utilisés seuls, ne modifient pas la proportion des ovocytes qui reprennent ou terminent leur méiose. Cependant ils préviennent les effets stimulants de IGF-I, IGF-II et EGF. Ces observations suggèrent que : (1) IGF-I, IGF-II et EGF sont des stimulateurs efficaces de la reprise et de l'achèvement de la maturation nucléaire chez le porc, (2) PKA est présente dans les ovocytes, et (3) les facteurs de croissance peuvent agir sur les ovocytes porcins par des mécanismes intracellulaires dépendant de la PKA.

IGF-I / IGF-II / EGF / protéine kinase A / méiose / porc

1. INTRODUCTION

Oocyte maturation may be regulated by growth factors produced by the surrounding follicular cells. This hypothesis is supported by the production of growth factors in ovarian cells and their accumulation in follicular fluid [4, 11, 38], by the presence of growth factor receptors in the oocyte [4], and by the ability of epidermal growth factor (EGF; rat: [5], mouse: [7], rabbit: [24], cow: [2, 15, 22–24], pig: [2, 6, 12, 31, 34]), insulin-like growth factor II (IGF-II, cow: [1]) and IGF-I (rabbit: [24, 42]; cow: [13, 23]) to promote nuclear maturation in cultured oocytes. Other authors found no influence of IGF-I on bovine [1, 14] or porcine [12, 31] oocytes. Thus although most reports describe meiosis-stimulating effects of growth factors, this requires further clarification.

Growth factors may influence ovarian cells indirectly, affecting a number of

gonadotropin receptors [19, 25] and ovarian response to gonadotropin [10], or directly, via growth factor receptors and related intracellular messengers. The intracellular mechanisms by which growth factors affect oocyte maturation are unknown. The mechanism may involve tyrosine kinase (TK) because TK activity is increased during porcine meiosis [16, 27] and genistein, a TK inhibitor, inhibits maturation of the porcine oocyte-cumulus complex [18]. In other experiments, however, genistein failed to prevent EGF [33]- and IGF-I [38]-induced cumulus expansion and oocyte maturation, suggesting that TK does not mediate growth factor effects on meiosis. Protein kinase C is probably not involved in this process [17, 37].

There is some data to suggest that oocyte maturation is regulated by the cAMP/protein kinase A (PKA) system but the evidence is not unequivocal. EGF increased cAMP formation in murine oocytes [7] and

in some [33] but not all [3] experiments on porcine oocytes. Activators of the cAMP/PKA system inhibited murine [9, 32] and rabbit [40, 41] oocyte maturation. Growth and maturation of the porcine oocyte-cumulus complex was respectively activated [29] and inhibited [17, 33] by stimulators and blockers of PKA. In other experiments, however, the maturation of porcine [3, 30] and bovine [37] oocytes was prevented by stimulators of cAMP/PKA. The involvement of the catalytic subunit of PKA in the mediation of GH/IGF-I effect was previously demonstrated on ovarian follicular cells [36], but not on oocytes. Downs and Hunzicker-Dunn [8] showed that different isoforms of PKA can have a paradoxical action on murine oocyte-cumulus complexes: the cAMP analogues-activators of type I regulatory subunit of PKA inhibited oocyte maturation. Therefore, the cAMP/PKA system is a hypothetical mediator of growth factor effect on oocytes, although mechanisms of PKA action on oocytes, as well as the interrelationships between cAMP/PKA and growth factors other than EGF, remain unknown. Oocyte PKA itself and the effects of growth factors on PKA within the oocytes have not been analyzed previously. Therefore, the role of PKA in mediating growth factor effects on oocytes requires further study.

The aim of our investigations was to determine (1) the influence of IGF-I, IGF-II and EGF on porcine oocyte nuclear maturation, (2) the presence of PKA within oocytes, and (3) the role of the cAMP/PKA system in mediating the effects of growth factors on porcine oocytes. In the present study we analyzed nuclear rather than cytoplasmic maturation of oocytes.

2. MATERIALS AND METHODS

2.1 Collection and culture of oocytes

Ovaries from non-cycling Slovakian white gilts, 180 days of age without visible

reproductive abnormalities, were obtained from a local abattoir. All experiments were performed in winter to avoid the influence of season. One hour after slaughter the oocytes were aspirated by syringe from ovarian follicles 2–8 mm in diameter. Haemorrhagic, cystic, presumptive preovulatory follicles (wider than 8 mm) and follicles with visible signs of atresia were excluded. The aspirated content of follicles was washed 3 times in TCM-199 (Sigma, St.-Louis, USA) supplemented with 10% FCS (Sigma). Oocytes were collected with a glass pipette, while small, pycnotic, vacuolated oocytes and oocytes without a developed cumulus investment were excluded from the experiments. Subsequently, oocytes were cultured in 5% CO₂ in air, at 37.5 °C under paraffin oil in 1 mL of TCM-199 supplemented with 10% heat inactivated FCS and 50 µg·mL⁻¹ antibiotic-antimycotic solution (Sigma). The following treatments were added to the medium of experimental groups: recombinant human IGF-I (Calbiochem, Lucerne, Switzerland; 10 ng·mL⁻¹), IGF-II, EGF (Sigma; both 10 ng·mL⁻¹), Rp-cAMPS (cAMP antagonist) or KT5720 (direct inhibitor of PKA; both 100 ng·mL⁻¹; Calbiochem-Novabiochem Corp. La Jolla, USA), a combination of IGF-I with EGF and combinations of IGF-I, IGF-II and EGF with Rp-cAMPS or KT5720 at the doses indicated above. It has previously been shown that these doses of the preparations have maximal effects on hormone release by porcine granulosa cells [35] and on porcine oocyte maturation [38]. Lyophilized IGF-I and IGF-II were reconstituted in 100 µL of 10 mM HCl, while KT5720 was dissolved in 50 µL of DMSO to reach concentrations of 1 mg·mL⁻¹. Immediately before the experiment, these stock solutions were dissolved in incubation medium so that the content of HCl or DMSO did not exceed 0.001% of medium. Other substances were dissolved immediately before experiment in the incubation medium mentioned above. Control medium contained none of these supplements. In the first series

of experiments (dynamics of nuclear maturation of oocytes), oocytes were cultured for 48 h and samples collected after 0, 12, 24, 36 and 48 h of incubation. In the second series of experiments (effects of growth factors and PKA inhibitors on oocyte maturation and PKA content), oocytes were collected after 40 h of culture (the first series of experiments indicated that this was sufficient time for the completion of nuclear maturation in more than 50% of oocytes).

2.1.1. Cytogenetical analysis

The collected oocytes were processed according to Tarkowski [39]. They were washed for 1 min in 0.9% Na citrate and after removal of cumulus oophorus by pipette were placed on microscopic slides, fixed with a mixture of pure methanol and glacial acetic acid (3:1), air-dried and stained with Giemza (Sigma) for determination of the meiotic stage as indicated by the structure of stained chromosomes. Oocytes which remained at the diplotene-dictyotene stage were considered as "not resuming meiosis"; oocytes at diakinesis, metaphase I, anaphase I, telophase I and metaphase II of meiosis were considered to be "resuming nuclear maturation"; oocytes which had reached metaphase II were classified as having completed pre-fertilization nuclear maturation. Oocytes with degenerated chromosomes (uncoiled, fragmented, aggregated or unspread) in which the stage of meiosis was difficult to determine were excluded from analysis (4–9% of the total population of oocytes in each group).

2.1.2. Immunocytochemical analysis

A fraction of each preparation was destined by washing in 70% ethanol and processed by immunocytochemistry [28] using primary mouse monoclonal antibodies against the regulatory subunit I (dilution 1:1000) and catalytic subunit C-alpha (dilution 1:250) of PKA (Transduction Laboratories, Lexington, USA). These antibodies

cross-react with the related subunits of human, dog, rat, bovine and porcine PKAs. The activity and specificity of these antibodies at these dilutions, as well as the ability of both Rp-cAMPS and KT5720 to block PKA immunoreactivity were confirmed prior to the experiments by Western blotting and immunocytochemical analysis of porcine granulosa cells (not shown). For visualization of PKA, a secondary polyclonal antibody was used against mouse IgG labeled with horseradish peroxidase (HPA) (SwAM Px, Sevac, Prague, Czech Republic; dilution 1:500) and DAB-reagent (Boehringer Mannheim GmbH, Mannheim, Germany; 10%). The oocytes treated with anti-mouse IgG-HPA and DAB, but not with anti-PKA primary antibodies, were used as negative control. These oocytes contained up to 10 grains of DAB per cell. Therefore, oocytes containing more than 10 grains of DAB per cell were defined as positively stained.

2.1.3. Protein gel electrophoresis and immunoblotting

A number of oocytes (30 per group) was lysed by repeated freezing-thawing and pipetting in 50 μ L of kinase lysis buffer (1% Triton X-100, 0.5% Igepal (PN-40), 5 mM EDTA, 20 μ g·mL⁻¹ phenylmethylsulfonyl fluoride, 10 μ g·mL⁻¹ aprotinin, 10 μ g·mL⁻¹ leupeptin, and 10 mM sodium ortovanadate in PBS, pH 7.5, all from Sigma). The lysates then were mixed 1:1 with electrophoretic buffer (0.0625 M Tris-base, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.003% bromophenol blue, all from Sigma), boiled at 95 °C for 3 min and subjected to SDS-polyacrilamide gel electrophoresis in 4% and 10% polyacrilamide in stacking and resolving gel, respectively, at a constant current of 25 mA according to Laemmli [20]. The samples were then transferred to nitrocellulose membrane ECL Hybond (Amersham International plc., Little Chalfont, UK) using a semi-dry trans-blotter (Bio-Rad Labs, Richmond, USA) during 1 h.

Endogenous peroxidase in samples was blocked by incubation in 3% H₂O₂ for 15 min, and non-specific binding of anti-serum was prevented by incubation in 5% blot-qualified BSA (Amersham International plc.) in TTBS (20 nM Tris-base, 137 nM NaCl, 0.1% Tween-20). Blocked membrane was probed with mouse monoclonal antibody against the catalytic subunit of PKA described above, at a dilution of 1:250. The membrane was then detected using secondary HPA-conjugated anti-rabbit IgG antibody, and visualized using ECL detection reagents and ECL Hyper-film (all from Amersham International plc.) according to the manufacturers instructions. Lysates of rat pituitary glands (provided by Amersham International plc.) and porcine ovarian follicles (5 cut follicles, 3 mm in diameter, washed free of follicular fluid using PBS, in 100 µL of kinase lysis buffer) acted as positive controls, while incubation medium without cells was used as a negative control. The molecular weights of fractions were evaluated using a molecular weight calibration kit (from 14.4 to 94.0 kD) from Serva (Heidelberg, Germany).

2.3. Statistics

Each experimental group was represented by 20–30 oocytes. Each experiment was performed on oocytes obtained from 20–40 animals. As a rule, all treatments were tested simultaneously in the same experiment. The data shown are means obtained in 3 to 6 replicate experiments performed on separate days using separate pools of cells. In the first series of experiments (maturation of oocytes during culture) the cytogenetical analysis was performed on 701 oocytes, whereas in the second series (effects of growth factors and PKA blockers), 1565 oocytes were analyzed. Immunocytochemical detection of the PKA catalytic subunit was performed on 119 oocytes, whilst the PKA regulatory subunit was identified in 147 oocytes from the second series of

experiments. Immunoblotting was performed on 540 oocytes from the second series of experiments. The coefficient of variation between experiments did not exceed 22%. Significant differences in the proportion of oocytes at different meiotic stages, or in PKA immunoreactivity content were evaluated by the Chi-square test. Differences from the control with $P < 0.05$ were considered significant.

3. RESULTS

3.1. Nuclear maturation of porcine oocytes during in vitro culture (series I)

It was observed that immediately after isolation and before culture, 96% of porcine oocytes were at the diplotene stage of meiosis. The remainder were at diakinesis-metaphase I. No oocytes were observed to have completed meiosis (at telophase-metaphase II) so far. After 12 and 24 h of culture, the number of oocytes which resumed meiosis had increased but very few reached completion. After 36 h of incubation only a few oocytes remained suspended at diplotene; 46% of the cells had completed nuclear maturation. After 48 h of culture 67% of oocytes had completed meiosis (Fig. 1).

3.2. Effects of growth factors on oocyte nuclear maturation (series II)

This series of experiments (Tab. I) demonstrated that all the growth factor treatments had a stimulatory influence on both the resumption and completion of meiosis. IGF-I, EGF and IGF-II reduced the percentage of oocytes which failed to resume meiosis and increased the proportion of oocytes completing meiosis. The combination of IGF-I + EGF stimulated completion of meiosis too.

3.3. Effects of PKA inhibitors on spontaneous and growth factor-induced oocyte maturation (series II)

Nor inhibitor of PKA (neither the cAMP antagonist Rp-cAMPS nor the direct PKA

blocker KT5720) substantially affected the resumption or completion of meiosis. Both PKA inhibitors prevented the effects of IGF-I or EGF on the resumption and completion of oocyte nuclear maturation. The effects of IGF-II were also prevented by Rp-cAMPS, whilst KT5720 enhanced the stimulating

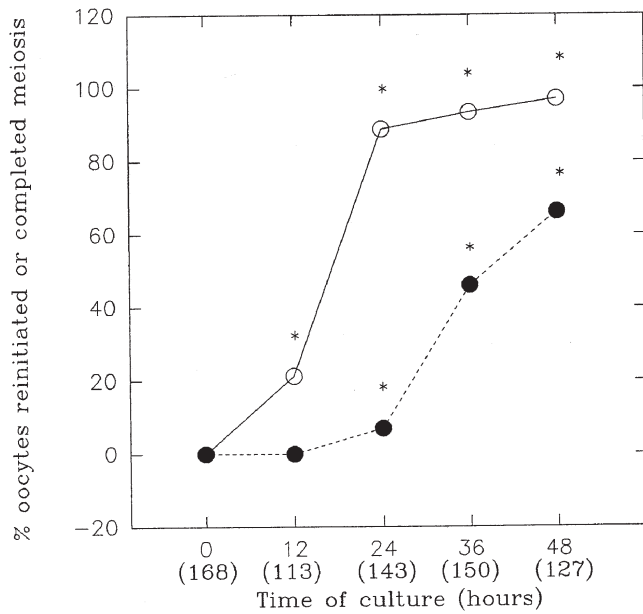


Figure 1. Changes in the proportion of porcine oocytes which resumed meiosis (white circles) and completed meiosis (black circles) during in vitro culture in TCM-199 supplemented with 10% FCS without treatments. The number of analyzed oocytes is indicated in brackets.

Table I. Effects of growth factors, inhibitors of PKA and their combinations on the nuclear maturation of porcine oocytes after 40 h culture.

Treatment	Number of analyzed oocytes	% oocytes	
		Not resumed meiosis	Completed meiosis
Control	109	15.6	37.6
Rp-cAMPS	156	10.3	41.0
KT5720	153	13.1	39.9
IGF-I	154	6.5*	72.7*
IGF-I + Rp-cAMPS	141	11.4	51.1
IGF-I + KT5720	136	20.6	43.4
EGF	117	4.3*	59.0*
EGF + IGF-I	63	7.9	57.2*
EGF + Rp-cAMPS	111	18.9	40.5
EGF + KT5720	102	8.6	39.2
IGF-II	118	7.6*	56.8*
IGF-II + Rp-cAMPS	96	15.6	39.6
IGF-I + KT5720 I	109	2.8*	44.0

* Significantly ($p < 0.05$) different from control (without additions).

effect of IGF-II on the resumption of meiosis but did not influence its effect on the completion of meiosis in porcine oocytes (Tab. I).

3.4. Effects of growth factors on PKA within oocytes (series II)

Immunocytochemical studies showed that many oocytes contained immunoreactivity corresponding to the regulatory and catalytic subunits of PKA. Furthermore, growth factors were able to increase the proportion of oocytes containing PKA (Tab. II). IGF-I increased the proportion of cells containing both the regulatory and catalytic subunits of PKA. IGF-II or EGF caused a slight increase in the percentage of oocytes with detectable amounts of the catalytic subunit of PKA but had no influence on the amount of regulatory subunit. Therefore, in further studies using immunoblotting, only the catalytic subunit of PKA was analyzed.

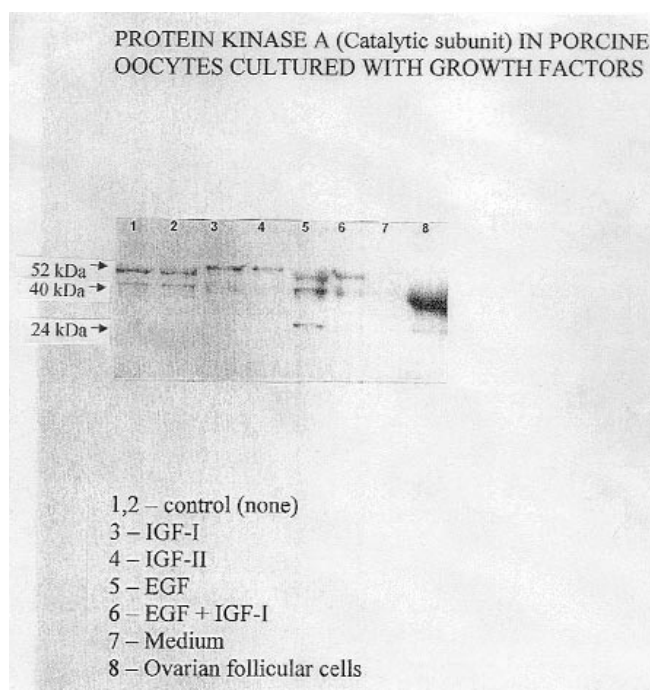
Immunoblotting (Fig. 2) confirmed the presence of the catalytic subunit of PKA in porcine oocytes. In the control and in most of the experimental groups this substance was represented by two immunoreactive fractions with an approximate MW of 52 kD (upper band) and 40 kD (middle band). Addition of IGF-I and IGF-II did not

Table II. Effects of growth factors on PKA content in porcine oocytes after 40 h culture.

Treatment	% oocytes contained	
	catalytic PKA subunit	regulatory PKA subunit
Control (40)	61.9	65.0
IGF-I (37)	77.8*	86.5*
IGF-II (28)	72.2*	64.3
EGF (42)	68.6	54.8

* Significantly ($p < 0.05$) different from control (without additions). The number of oocytes analyzed is indicated in brackets.

Figure 2. Effect of growth factors on protein kinase A (catalytic subunit) in porcine oocytes after 40 h culture in TCM-199 supplemented with 10% FCS (SDS-PAGE and Western immunoblotting). Groups: 1, 2 – oocytes cultured without treatments (control); 3 – oocytes cultured with IGF-I; 4 – oocytes cultured with IGF-II; 5 – oocytes cultured with EGF; 6 – oocytes cultured with EGF + IGF-I; 7 – medium incubated without cells (blank control); 8 – whole ovarian follicle (positive control).



substantially affect the expression of these fractions, although an insignificant trend towards a mobility shift of the 52 kD fraction and a reduction in the intensity of the 40 kD fraction did occur in these groups. EGF stimulated the expression of both forms (52 kD and 40 kD) of the catalytic subunit of PKA, but not the mobility shift of the 52 kD band. Furthermore, it induced the appearance of the 3rd isoform, the smallest fraction of the catalytic subunit of PKA with an MW of about 24 kD. The presence of IGF-I prevented the appearance of this 3rd, EGF-induced PKA isoform: the PKA profile in oocytes treated with a combination of EGF + IGF-I did not differ qualitatively from that in the control or IGF-I-treated cells. The lysate of ovarian follicles contained only well-expressed middle forms (40 kD) and less visible small forms (24 kD), but no large form (52 kD) of the catalytic subunit of PKA, which was characteristic of oocytes. Blank control (incubation medium) contained no PKA immunoreactivity.

4. DISCUSSION

The results presented in Figure 1 and Table I demonstrate that although we used oocytes from prepubertal animals killed in a slaughterhouse, which potentially reduced developmental competence, the majority of oocytes in our experiments were able to resume and complete meiosis and respond to growth factors and pharmacological agents. Furthermore, our present data are consistent with other reports (see Introduction) on the ability of IGF-I and EGF to stimulate oocyte nuclear maturation in various mammalian species, although they do not correspond with the data of some authors [12, 31] on the absence of an IGF-I effect on this process. Moreover, our observations are the first to demonstrate the involvement of IGF-II in the regulation of oocyte maturation in pigs. Growth factors, especially IGF-I, EGF or their combination, can be used to improve the production of matured

oocytes for fertilization and embryo production.

There is evidence that in non-ovarian cells, growth factors exert their effects mainly via tyrosine kinase-coupled receptors [21, 26]. Several experiments with tyrosine kinase blockers have indicated the involvement of tyrosine kinase in the action of growth factors on the maturation of porcine oocytes [16, 18, 27], but in our previous experiments tyrosine kinase blockers failed to influence the stimulatory effect of IGF-I on porcine oocyte maturation [38]. Regulation of spontaneous and IGF-I-stimulated maturation of oocytes in pigs and other species by stimulators [8, 9, 29, 30, 32, 37, 40, 41] or inhibitors [3, 17] of PKA provides indirect evidence that the cAMP/PKA system may be another mediator of growth factor action on oocytes. Direct detection of PKA within oocytes, and the influence of growth factors on PKA has, however, not been reported previously.

The presence of PKA in porcine oocytes was demonstrated in our experiments using both Western blotting and immunocytochemistry. Western blotting showed the existence of at least 2 isoforms of the catalytic subunit of PKA in porcine oocytes, and these were distinct from the PKA profile in ovarian follicles. The 40 kDa fraction is probably the standard form of PKA present in all the cells studied. The causes and significance of PKA polymorphism observed in our experiments is unknown. The least expressed 24 kDa fraction of PKA (present both in oocytes and in follicular cell lysate) can contain fragments-products of PKA degradation. The 52 kDa form may be the product of a tissue-specific mobility shift induced by phosphorylation, glycosylation and other modifications of PKA molecules induced by different agents, although the non-specific binding of antiserum against PKA by other peptides present in oocytes must not be completely excluded either.

The present observations are the first demonstration that different growth factors

are able to change the proportion of cells containing PKA as well as the pattern of PKA within oocytes. Data from immunocytochemistry demonstrated that all growth factors increased the percentage of cells containing PKA above the sensitivity limit of the method. The role of the regulatory subunit of PKA in the control of murine meiosis has previously been demonstrated [8]. Table II and Figure 2 suggest that the main target for growth factors in porcine oocytes is the catalytic subunit rather than the regulatory subunit of PKA: all three growth factors affected the catalytic subunit of PKA, while the regulatory subunit was regulated only by IGF-I. The effect of growth factors on PKA subunits in porcine oocytes resembles the effect of GH on bovine ovarian cells, where GH activated the catalytic subunit, but not the regulatory subunit of PKA [36]. Data from immunoblotting showed that growth factors are also able to affect the content and forms of catalytic subunits of PKA: EGF stimulated the expression of the 52 kD and 40 kD isoforms and induced the formation of the 24 kD isoform. IGF-I and IGF-II, in contrast to EGF, induced a mobility shift of the 52 kD band. In addition, IGF-II reduced the expression of the 40 kDa fraction, while IGF-I prevented EGF-induced formation of the 24 kD isoform PKA. Thus, all three growth factors were able to affect both quantitative and qualitative characteristics of the catalytic subunit of PKA, but the growth factors produced different patterns of effects: EGF changed the expression of all three forms of enzyme, IGF-II affected the 52 and 40 kD bands, while IGF-I was able to influence only the 52 kD fraction and to modify EGF effect on the 24 kD fraction of the catalytic subunit of PKA (Fig. 2). In contrast to IGF-II and EGF, IGF-I was able to affect the expression of not only the catalytic subunit, but also the regulatory subunit of PKA (Tab. II). Data from both immunocytochemistry and immunoblotting suggest that all three of the growth factors studied affect PKA, but that

the mechanisms underlying their effects on PKA may be distinct and even antagonistic.

Immunocytochemical data demonstrated the high variability between oocytes in their PKA content: some cells contained the latter, but in other cells, even after treatment with growth factors, PKA content remained below the level of sensitivity (10 grains of DAB per cell). It cannot be excluded that the PKA content determines the influence of cells on growth factors, since the oocytes with low PKA levels had a reduced ability to mature or respond to growth factors. Nevertheless, these processes may not depend on the total amount of PKA within oocytes but rather on the amount or type of bioactive PKA or its isoforms [8].

Although PKA in our experiments was analyzed after oocytes resumed or completed meiosis, the ability of growth factors to stimulate both PKA and nuclear maturation, as well as the ability of PKA inhibitors to prevent the effects of IGF-I, IGF-II and EGF, suggest that PKA can be an intracellular mediator of growth factor action on the oocyte. Thus, previous suggestions that PKA is a possible mediator of EGF action (see Introduction) are confirmed. The current data are the first to demonstrate that PKA can mediate the action of IGF-I and IGF-II as well as EGF. The most reliable evidence for the involvement of PKA in growth factor action is available with respect to IGF-I: this growth factor stimulated the accumulation of both PKA subunits and its effect could be prevented by both types of PKA inhibitor. In the case of EGF and IGF-II, inhibition by PKA antagonists was not consistently observed, suggesting the existence of one or several intracellular mechanisms other than PKA. Note also that these observations only partially correspond with reports of potent meiosis-inhibiting activity in PKA inhibitors [17]. In our experiments, KT5720 inhibited the completion but not the resumption of oocyte maturation; in other cases both Rp-cAMP and KT5720 given alone had no effect.

Nevertheless, the blockade of growth factor action by these substances suggests that they are specific rather than non-specific modulators of growth factor-induced processes and that active PKA is required for IGF-I, IGF-II and EGF to influence oocyte maturation.

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REFERENCES

- [1] Carolan C., Lonergan P., Monget P., Mermillod P., Role of insulin family of growth factors in bovine oocyte maturation (IVM) and embryo development (IVC) in vitro, *J. Reprod. Fertil.* 15 (1995) 71.
- [2] Coskun S., Sanbuissho A., Lin Y.C., Rikhisa Y., Fertilizability and subsequent developmental ability of bovine oocytes matured in medium containing epidermal growth factor (EGF), *Theriogenology* 36 (1991) 485–494.
- [3] Coskun S., Lin Y.C., Mechanism of action of epidermal growth factor-induced porcine oocyte maturation, *Mol. Reprod. Dev.* 42 (1995) 311–317.
- [4] Danforth D.R., Endocrine and paracrine control of oocyte development, *Am. J. Obstet. Gynecol.* 172 (1995) 747–752.
- [5] Dekel N., Sherizly I., Epidermal growth factor induces maturation of rat follicle-enclosed oocytes, *Endocrinology* 116 (1985) 406–409.
- [6] Ding J., Foxcroft G.R., Epidermal growth factor enhances oocyte maturation in pigs, *Mol. Reprod. Dev.* 39 (1994) 30–40.
- [7] Downs S.M., Daniel S.A.J., Eppig J.J., Induction of maturation in cumulus cell-enclosed mouse oocytes by FSH and epidermal growth factor. Evidence for a positive stimulus of somatic cell origin, *J. Exp. Zool.* 245 (1988) 86–96.
- [8] Downs S.M., Hunzicker-Dunn M., Differential regulation of oocyte maturation and cumulus expansion in the mouse oocyte-cumulus complex by site-selective analogs of cyclic adenosine monophosphate, *Dev. Biol.* 172 (1995) 72–85.
- [9] Eppig J.J., Mammalian oocyte development, in: Hillier S.G. (Ed.), *Ovarian Endocrinology*, Blackwell Science Publisher, Oxford, 1991, pp. 107–128.
- [10] Endo K., Atlas S.J., Rone J.D., Zanagnolo V.L., Kuo T.C., Dharamarajan A.M., Wallach E.E., Epidermal growth factor inhibits follicular response to human chorionic gonadotropin: possible role of cell to cell communication in the response to gonadotropin, *Endocrinology* 130 (1992) 186–192.
- [11] Giudice L.C., Insulin-like growth factors and ovarian follicular development, *Endocr. Rev.* 13 (1992) 641–668.
- [12] Grupen Ch.G., Nagashima H., Nottle M.B., Role of epidermal growth factor and insulin-like growth factor-I on porcine oocyte maturation and embryonic development in vitro, *Reprod. Nutr. Dev.* 9 (1997) 571–575.
- [13] Harper K.M., Brackett B.G., Enhanced bovine oocyte quality after in vitro maturation (IVM) with insulin-like growth factor-I (IGF-I) and gonadotropins, *Biol. Reprod.* 46 (1992) 67.
- [14] Harper K.M., Brackett B.G., Bovine blastocyst development after in vitro maturation in a defined medium with epidermal growth factor and low concentrations of gonadotropins, *Biol. Reprod.* 48 (1993) 409–416.
- [15] Im K.S., Park K.W., Effects of epidermal growth factor on maturation and development of bovine follicular oocytes, *Theriogenology* 44 (1995) 209–216.
- [16] Inoue M., Naito K., Aoli F., Toyoda Y., Sato E., Activation of mitogen-activated protein kinase during meiotic maturation in porcine oocytes, *Zygote* 3 (1995) 265–271.
- [17] Jung T., Lee C., Moor R.M., Effects of protein kinase inhibitors on pig oocyte maturation in vitro, *Reprod. Nutr. Dev.* 32 (1992) 461–473.
- [18] Jung T., Fulka J. Jr., Lee C., Moor R.M., Effects of the protein phosphorylation inhibitor genistein on maturation of pig oocytes in vitro, *J. Reprod. Fertil.* 98 (1993) 529–535.
- [19] Kanzaki M., Hattori M.-A., Horiuchi R., Kojima I., Co-ordinate actions of FSH and insulin-like growth factor-I on LH receptor expression in rat granulosa cells, *J. Endocrinol.* 141 (1994) 301–308.
- [20] Laemmli U.K., Cleavage and structure of proteins during the assembly of the head of bacteriophage T4, *Nature (London)* 227 (1970) 680–685.
- [21] LeRoith D., Werner H., Beitner-Johnson D., Roberts C.T., Molecular and cellular aspects of the insulin-like growth factor I receptor, *Endocr. Rev.* 16 (1995) 143–163.
- [22] Lonergan P., Carolan C., van Longendonck A., Donnay I., Khatir H., Mermillod P., Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development in vitro, *Biol. Reprod.* 54 (1996) 1420–1429.

- [23] Lorenzo P., Illera M.J., Illera J.C., Illera M., Enhancement of cumulus expansion and nuclear maturation during bovine oocyte maturation in vitro by the addition of epidermal growth factor and insulin-like growth factor I, *J. Reprod. Fertil.* 101 (1994) 697–701.
- [24] Lorenzo P.L., Rebollar P.G., Illera M.J., Illera J.C., Illera M., Stimulatory effect of insulin-like growth factor I and epidermal growth factor on the maturation of rabbit oocytes in vitro, *J. Reprod. Fertil.* 107 (1996) 109–117.
- [25] Maruo T., Hayashi M., Matsuo H., Ueda Y., Morikawa H., Moshizuki M., Comparison of the facilitative roles of insulin and insulin-like growth factor-I in the functional differentiation of granulosa cells: in vitro studies with the porcine model, *Acta Endocrinol. (Copenhagen)* 117 (1988) 230–240.
- [26] McInnes C., Sykes B.D., Growth factor receptors: structure, mechanism, and drug discovery, *Biopolymers* 43 (1997) 339–366.
- [27] Moor R., Hutchings A., Hawkins C., Jung T., Lauria A., Towards a molecular understanding of meiosis in domestic animals, in: Gandolfi F. (Ed.), *Embryonic Development and Manipulation in Animal Production: Trends in Research and Applications*, 1992, pp. 1–15.
- [28] Osborn M., Isenberg S., Immunocytochemistry of frozen and of paraffin tissue sections, in: Celis J.E. (Ed.), *Cell Biology: A Laboratory Handbook*, Academic Press, San Diego - New York - Boston - London - Sydney - Tokyo - Toronto 2, 1994, pp. 361–367.
- [29] Petr J., Tepla O., Groholova R., Jilek F., Inhibition of meiotic maturation in growing pig oocytes by factor(s) from cumulus cells, *Reprod. Nutr. Dev.* 34 (1994) 149–156.
- [30] Petr J., Tepla O., Rozinek J., Jilek F., Effect of testosterone and dibutyl cAMP on the meiotic competence of pig oocytes in various size categories, *Theriogenology* 46 (1996) 97–108.
- [31] Reed M.L., Estrada J.L., Illera M.J., Petters R.M., Effect of epidermal growth factor, insulin-like growth factor-I and dialyzed porcine follicular fluid on porcine oocyte maturation in vitro, *J. Exp. Zool.* 266 (1993) 74–78.
- [32] Sato E., Koide S.S., Biochemical transmitters regulating the arrest and resumption of meiosis in oocytes, *Int. Rev. Cytol.* 106 (1987) 1–33.
- [33] Singh B., Signal transduction pathways of epidermal growth factor (EGF)-induced porcine cumulus expansion and oocyte maturation, *Biol. Reprod.* 43 (1993) 102.
- [34] Singh B., Barbe G.J., Armstrong D.T., Factors influencing resumption of meiotic maturation and cumulus expansion of porcine oocyte-cumulus cell complexes in vitro, *Mol. Reprod. Dev.* 36 (1993) 113–119.
- [35] Sirotkin A.V., Inter-relationships between non-peptide hormones and cyclic nucleotides within cultured porcine granulosa cells, *J. Endocrinol.* 150 (1996) 343–348.
- [36] Sirotkin A.V., Makarevich A.V., GH regulates secretory activity and apoptosis in cultured bovine granulosa cells through the activation of the cAMP/protein kinase A system, *J. Endocrinol.* 163 (1999) 317–327.
- [37] Sirotkin A.V., Mikhaeljan H.G., Golubev A.K., Bulla J., The involvement of peptide hormones and related intracellular messengers in the regulation of bovine oocyte maturation in vitro, *Živ. Vyroba* 40 (1995) 249–255.
- [38] Sirotkin A.V., Taradajnik T.E., Makarevich A.V., Bulla J., Effect of follicular cells, IGF-I and tyrosine kinase blockers on oocyte maturation, *Anim. Reprod. Sci.* 51 (1998) 333–344.
- [39] Tarkowski A.K., An air-drying method for chromatin preparation for mouse eggs, *Cytogenetics* 5 (1966) 394–400.
- [40] Yoshimura Y., Nakamura Y., Oda T., Ando M., Ubukata Y., Karube M., Koyama A., Yamada H., Induction of meiotic maturation of follicle-enclosed oocytes of rabbits by a transient increase followed by an abrupt decrease in cyclic AMP concentration, *J. Reprod. Fertil.* 95 (1992) 803–812.
- [41] Yoshimura Y., Hosoi Y., Atlas S.J., Ghodgaonkar R., Dubin N.H., Dharmarajan A.M., Wallach E.E., Inhibition of gonadotropin-induced ovulation in rabbits by perfusion with dibutyl cAMP via reduction of ovarian prostaglandin production, *J. Reprod. Fertil.* 101 (1994) 207–212.
- [42] Yoshimura Y., Ando M., Nagamatsu S., Iwashita M., Adashi T., Sueoka K., Miyazaki T., Kuji N., Tanaka M., Effects of insulin-like growth factor-I on follicle growth, oocyte maturation, and ovarian steroidogenesis and plasminogen activator activity in the rabbit, *Biol. Reprod.* 55 (1996) 152–160.