PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 73 (2000) 123–133

The Journal of
Steroid Biochemistry
&
Molecular Biology

www.elsevier.com/locate/jsbmb

The role of IGF-I, cAMP/protein kinase A and MAP-kinase in the control of steroid secretion, cyclic nucleotide production, granulosa cell proliferation and preimplantation embryo development in rabbits

A. Makarevich*, [1], A. Sirotkin, P. Chrenek, J. Bulla, L. Hetenyi

Research Institute of Animal Production, Hlohovska 2, 94992 Nitra, Slovak Republic

Received 30 August 1999; accepted 6 March 2000

Abstract

The aim of this study was to investigate the actions of insulin-like growth factor I (IGF-I) on the secretory and proliferative functions of rabbit ovarian cells and on early embryogenesis. It was found that addition of IGF-I at a lower concentration (1 ng/ml) stimulated progesterone secretion by cultured rabbit granulosa cells, whilst higher concentrations of IGF-I (10, 100 ng/ml) were inhibitory. IGF-I had no effect on estradiol secretion. Cyclic AMP secretion was slightly increased after addition of IGF-I at 10 ng/ml, but not by higher concentrations. Cyclic GMP secretion was stimulated by IGF-I at 100 ng/ml only. A blocker of protein kinase A, Rp-cAMPS, did not alter progesterone and estradiol secretion but did prevent the action of IGF-I on progesterone secretion. An immunocytochemical study demonstrated that IGF-I significantly increased the proportion of proliferating cell nuclear antigen-positive (PCNA-positive) cells. Rp-cAMP did not change cell proliferation but partially prevented the proliferation-stimulating effect of IGF-I. IGF-I (100 ng/ml) significantly increased the proportion of divided zygotes and the number of embryos reaching the morula/blastocyst stage. Blockers of PKA, Rp-cAMPS and KT5720, reversed the effects of IGF-I on zygote cleavage and embryo development. Addition of IGF-I (100 ng/ml) significantly increased MAPK within the cells (proportion showing immunoreactivity to ERK-1 and ERK-3 antibodies and intensity of a 42 kDa band related to ERK-2). Rp-cAMPS suppressed the basal ERK-2 immunoreactivity but not that of ERK-1 or ERK-3. It completely inhibited the IGF-I-induced activation of ERK-3 but not that of ERK-1 or ERK-2. This in vitro study demonstrates that IGF-I is a potent stimulator of ovarian secretion, proliferation and embryogenesis in rabbit. Its effects are mediated by cAMP/PKA- and, probably by, MAPK-dependent intracellular mechanisms.

Keywords: PCNA; ERKs; cAMP; cGMP; Progesterone; Estradiol; Zygote

1. Introduction

Recent studies show that growth factors can play a key role in the control of reproduction. The ovary is a site of both production and action of insulin-like growth factor I (IGF-I) [1]. The growth factor may be involved in autocrine and paracrine control of ovarian steroidogenesis, proliferation and differentiation[2–4]. IGF-I is a potent stimulator of progesterone biosynthesis and secretion in cultured bovine [5], porcine [6] and ovine [7] granulosa cells, although it has no such effect on hen [8] or human [9] cells or on the perfused rabbit ovary [10]. Data concerning the effects of IGF-I on ovarian estrogen production are incomplete. Thus, IGF-I stimulated estradiol production by cultured porcine [11], rat [12,13] and human [9] ovarian cells and by perfused rabbit ovaries [10] but not by hamster

* Corresponding author. Agricultural Research Centre of Finland, 31600 Jokioinen, Finland.

E-mail address: alexander.makarevich@mtt.fi (A. Makarevich).

[1] Tel.: +421-87-546-335; fax: +421-87-546-361.

0960-0760/00/$ - see front matter
PII: S0960-0760(00)00067-4

proestrous follicles [14]. Both stimulatory [15] and inhibitory [16] effects have been reported with bovine granulosa cells. The effects of IGF-I on progesterone and estradiol release by rabbit granulosa cells, have not been fully studied.

IGF-I is a recognized stimulator of oocyte maturation and embryogenesis in mouse [17], buffalo [18], pig [19], cattle [20] and rabbit [21]. It stimulates proliferation in ovine [7] and bovine [5] granulosa cells. Moreover, opposite interrelationship between apoptosis in early embryos and their proliferation was found [21].

The precise mechanisms of growth factor action in the reproductive system are still unclear. It was observed that IGF-I action on ovarian function was associated with the activation of tyrosine kinase (human [22], rat [23], bovine [24]), cAMP/protein kinase A (mouse [25]; pig [26]) or protein kinase C (bovine [24]). The effects of IGF-I on other kinases (MAP etc.) within the ovary have not yet been studied. There is no direct evidence that protein kinases A, C and MAP are the putative mediators of IGF-I action but such evidence could be obtained by studying the ability of kinase blockers to prevent IGF-I action.

The aims of this study were to investigate the effects of IGF-I on secretory and proliferative functions of cultured rabbit ovarian granulosa cells and on embryogenesis, and to determine which protein kinases mediate these effects. The effects of IGF-I and PKA blockers on (a) progesterone, estradiol, cAMP and cGMP secretion, (b) proliferation by and presence of MAP kinase in granulosa cells, and (c) on preimplantation embryo development, were studied.

2. Experimental

2.1. Isolation, culture and analysis of rabbit ovarian granulosa cells and zygotes

Periovulatory ovaries from New Zealand White rabbits, 4 months of age, were transported to the laboratory at ambient temperature in a glass container within 1 h of slaughter at a local rabbit farm. They were washed in Dulbecco's PBS (D'PBS) with 1% antibiotic–antimycotic solution (Sigma, St.Louis, USA), placed in 100 mm diameter culture dishes (Gama, Çeské Budejovice, Czech Republic) and dissected using a multiblade knife. The tissue suspension was passed through a steel sieve to separate large pieces and cellular fragments. The cell filtrate was washed three times in D'PBS solution and granulosa cells were purified from cell debris and blood cells by centrifugation in a gradient of Percoll (Sigma). Granulosa cells were aspirated from the Percoll fraction and rinsed twice in sterile incubation medium. After the final centrifugation, the cells were resuspended in the incubation medium DME/F12 supplemented with 10% fetal calf serum (FCS, University of Veterinary Medicine, Brno, Czech Republic) and 1% antibiotic–antimycotic solution. The cell concentration was determined with a haemocytometer and then adjusted to 1×10^6 cells/ml by dilution with incubation medium. Cell viability was determined by Trypan blue staining and found to be in the range of 65–80%. Two millilitre aliquots of granulosa cell suspension (1×10^6 cells/ml) intended for RIA or Western immunoblotting were cultured in Falcon 24-well plates (Becton Dickinson, Lincoln Park, USA), whilst 300 μl aliquots of cell suspension intended for immunocytochemical analysis were incubated in Lab-Tek chamber-slides (Nunc, Naperville, USA) at 37°C and 5% CO_2 in humidified air until a 70% confluent monolayer was formed.

After 2–3 days, the medium was replaced with fresh serum-free medium of the same composition, supplemented with recombinant human IGF-I (>97% purity by SDS-PAGE, at 0, 1, 10, 100, 1000 ng/ml medium; Calbiochem Lucerne, Switzerland) either alone or in combination with a PKA blocker Rp-cAMPS at 50 nM. In our pre-experimental study this concentration was showed to be enough to suppress PKA in cultured cells in situ (immunocytochemistry using antibodies against R- and C-subunits), as well as in cell lyzates (Western-blotting using antiserum against PKA C-subunit) (data not shown). A phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine; Calbiochem; 1 μg/ml) was added to cultures intended for cyclic nucleotide analysis. After 2 days culture, the medium from plate wells was gently aspirated and frozen at −18°C to await RIA. Cultured cells in 24 well plates were rinsed twice in serum-free D'PBS, lyzed by adding ice-cold kinase lysis buffer (1% Triton X-100, 0.5% Igepal NP-40, 5 mM EDTA, 20 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotonin, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 10 mM sodium ortovanadate in PBS, pH 7.5, all from Sigma, 50 μl/well) and frozen at −18°C to await gel electrophoresis and immunoblotting. Chamber-slides were washed three times in PBS, fixed overnight at −5°C with a pure acetone (Lachema, Brno, Czech Republic), air-dryed and kept at 4°C to await immunocytochemical analysis.

Zygotes were isolated from the oviducts of New Zealand White, 4 months of age, superovulated with i.m. injection of 80IU PMSG (Sergon, Bioveta, Ivanovice na Hane, Czech Republic). Seventy-two hours after PMSG treatment, they were mated twice with a male of proven fertility and immediately injected i.v. with 160 IU HCG (Praedyn, Leçiva, Praha, Czech Republic). Zygotes were recovered from the oviducts of slaughtered animals at 19 h after mating by flushing with 1 × D'PBS, containing 5% FCS. Parthenogenetic

zygotes were excluded after visual inspection of the presence of both pronuclei. Selected zygotes were put into four well culture dishes (Nunc, Roskilde, Denmark) at 20–30 zygotes per 50 μl drop of DME/F12 nutrient mixture (Sigma) supplemented with 1% BSA concentrate (ICP, Auckland, New Zealand) and treatments (described below) and cultured under a paraffin oil at 39°C and 5% CO_2.

The following treatment additions were made:

1. Recombinant human IGF-I (100 ng/ml; Calbiochem);
2. IGF-I (100 ng/ml) in combination with PKA blockers (Rp-cAMPS, 50 nmol or KT5720, 5 μg/ml; Calbiochem);
3. PKA blockers alone at the above concentrations.

Stages of embryogenesis at 96 h of culture were evaluated according to the number and position of blastomeres, using a light microscope. To exclude fragmented zygotes and embryos, cells were fixed in methanol–acetic acid (3:1 v/v) and stained with Giemsa dye (Sigma) according to Tarkowski [27] to detect the presence and quality of nuclei within each blastomere.

All chemicals used were of research grade and, with the exception of KT5720, were finally dissolved in the incubation medium just prior to use. KT5720 was initially dissolved in a small volume of DMSO (Serva, Heidelberg, Germany) to a concentration 100 μg/μl; this stock solution was then gradually dissolved in the culture medium under conditions of subdued direct light. Simultaneously, the same volume of DMSO without KT5720 was dissolved in the culture medium and added to the control group. The final DMSO content in the medium did not exceed 0.01%.

2.2. Radioimmunoassays (RIA)

Concentrations of growth factor, steroid hormones and cyclic nucleotides were determined in duplicate 25–100 μl samples of ovarian cell-conditioned media. IGF-I (after acid–ethanol extraction) and estradiol were analysed using commercial IRMA/RIA kits from DSL (Webster, Texas, USA). Progesterone, cyclic AMP and cyclic GMP (after succinylation) were measured using RIA kits from Immunotech (Marseille, France). Assay characteristics are summarized in Table 1. Cross-reactivity of Rp-cAMPS and KT5720 with each specific antiserum was non-detectable.

2.3. SDS-PAGE and Western immunoblotting

Frozen-thawed lysates of granulosa cells were mixed 1:1 with electrophoretic buffer (0.0625 M Tris-base, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.003% bromophenol blue, pH 6.8; all from Sigma), boiled at 95°C for 3 min and electrophoresed in 4% (stacking gel) and 10% (resolving gel) polyacrylamide at 25 mA constant current according to Laemmli [28]. The samples from the gel were transferred to nitrocellulose membranes (ECL Hybond, Amersham International, Little Chalfont, UK) using a semi-dry transblotter (Bio-Rad Labs, Richmond, USA) for 1 h at 0.8 mA/cm^2 of membrane. Endogenous peroxidase in samples was quenched by incubation in 3% H_2O_2 for 15 min. Non-specific binding of antiserum was blocked by incubation in 5% blot-qualified BSA (Amersham) in TTBS (20 nM Tris-base, 137 nM NaCl, 0.1% Tween-20, pH 7.5). Blocked membranes were probed for 1 h with mouse monoclonal antibody against the ERK-2 (IgG 2b, clone 33, Transduction Laboratories, Lexington, USA, dilution 1:4000). This antibody recognizes the active form of ERK-2 in human, dog, rat, mouse, chick, frog and rabbit cells, but shows slight cross-reactivity to ERK-1. Membranes were then incubated with secondary horseradish peroxidase-conjugated anti-mouse IgG antiserum, and positive signals were visualized using ECL detection reagents and ECL

Table 1
Characteristics of RIAs/IRMAs used in the study

Assay	Cross-reactivity of antiserum	Sensitivity of assay	Coefficient of variation (%)	
			Intraassay	Interassay
IGF-I	< 0.01% to IGF-II, insulin, proinsulin and GH	27 pg/ml	< 3.4	< 8.2
Progesterone	< 0.04% to cortizol, danazol, cortizone, estradiol, estriol DHEA; <1% to pregnenolone, androstenedione, dihydroprogesterone	0.03 ng/ml	< 8	< 15.6
Estradiol	< 0.01% to DHEA, progesterone, cortisol, danazol, androsterone, testosterone, androstene-dione, corticosterone, cortisone	6.5 pg/ml	< 9.4	< 19.2
Cyclic AMP	With ScAMP — 1, cAMP — 15, cGMP — 4×10^4, 5′AMP — 7×10^4, ATP — 7×10^3, ScGMP — 2×10^4,	0.2 nM/tube	< 11	< 16
Cyclic GMP (succinylated)	Succinyl cGMP — 100%, succinyl cAMP — 0.003%, cyclic GMP — 1.9%, cyclic AMP — 0.0001%	10 pM/tube	Data not provided	Data not provided

Hyper-film (all from Amersham). A human fibroblast cell lysate (Transduction Laboratory), at 10 μg/10 μl, containing significant amounts of ERK-2, was used as a positive control. The molecular weights of fractions were evaluated using a molecular weight calibration kit (14.4–94.0 kD; Serva, Heidelberg, Germany).

2.4. Immunocytochemical analysis

Cell proliferation was detected immunocytochemically [29] in granulosa cells plated in the chamber-slides, using mouse monoclonal antibody Ab-1, clone PC10 (Oncogene Research Products, Cambridge, MA, USA) against proliferating cell nuclear antigen (PCNA) at a dilution of 1: 40. The presence of ERK-1 and ERK-3 in the cells was detected using mouse monoclonal antibodies IgG1, clone MK12 (1:500) and IgM clone 30 (1:250), respectively. The activity and specificity of these antibodies at these dilutions were confirmed prior to experiment by Western blotting (data not shown). For the visualization of the binding sites of primary antibodies, a secondary polyclonal horseradish peroxidase-conjugated antiserum against mouse IgG (Sevac, Prague, Czech Republic; dilution 1:500) and DAB-reagent (Boehringer Mannheim GmbH, Mannheim, Germany; 0.1% in PBS) were used. Cells treated with secondary antibody and DAB, omitting primary antibodies, were used as a negative control. The presence of PCNA and ERKs immunoreactivity in the cells was evaluated by light microscopy.

2.5. Statistics

Each experimental group was represented by four culture wells or chambers of cultured granulosa cells and by 55–100 rabbit zygotes. Proportions of cells containing PCNA and ERK immunoreactivity were calculated on the basis of inspection of a minimum 1000 cells per group. The data shown are means of values obtained in four to six separate experiments performed on different days using separate pools of cells, each obtained from 20–40 animals. In RIA, the values of blank control (cell-free medium) were subtracted from the value determined in cell-conditioned medium to exclude the non-specific background. The rates of substance secretion were calculated per 10^6 viable cells/day. Significant differences between the experiments were evaluated using two-way ANOVA. When effects of treatments were revealed, data from the experimental and control groups were compared by Duncan's multiple range test. Significant differences between the groups in the developmental rates of cultured rabbit zygotes were evaluated by the Chi-square test. Differences from control at $p < 0.05$ were considered as significant.

3. Results

3.1. Effects of IGF-I and protein kinase A blockade on secretory activity of cultured rabbit granulosa cells

In preliminary experiments, rabbit granulosa cells released detectable amounts of IGF-I, progesterone, estradiol, cAMP and cGMP into the incubation medium (Table 2).

Addition of exogenous IGF-I affected the secretion of progesterone and cyclic nucleotides into the culture medium. The effect of IGF-I on progesterone was biphasic: it stimulated secretion at lower concentration (1 ng/ml), but dramatically inhibited secretion at higher concentrations (10, 100 ng/ml, $p < 0.05$; Fig. 1). No effect of IGF-I on estradiol secretion was observed (Fig. 2). Cyclic AMP secretion was increased after addition of IGF-I at 10 ng/ml ($p < 0.05$; Fig. 3) but not at other concentrations. Cyclic GMP secretion was stimulated by IGF-I at 100 ng/ml, ($p < 0.05$), but not at lower concentrations (Fig. 4).

Blockade of protein kinase A by Rp-cAMPS did not change the basal level of steroid secretion but it completely prevented the IGF-I effect on progesterone secretion (Fig. 1). Rp-cAMPS in combination with IGF-I (100 ng/ml), induced an insignificant increase in estradiol secretion (Fig. 2). The amount of estradiol released under the influence of IGF-I + Rp-cAMPS differed significantly ($p < 0.01$) from that in the presence of IGF-I alone, but not from that in the untreated control.

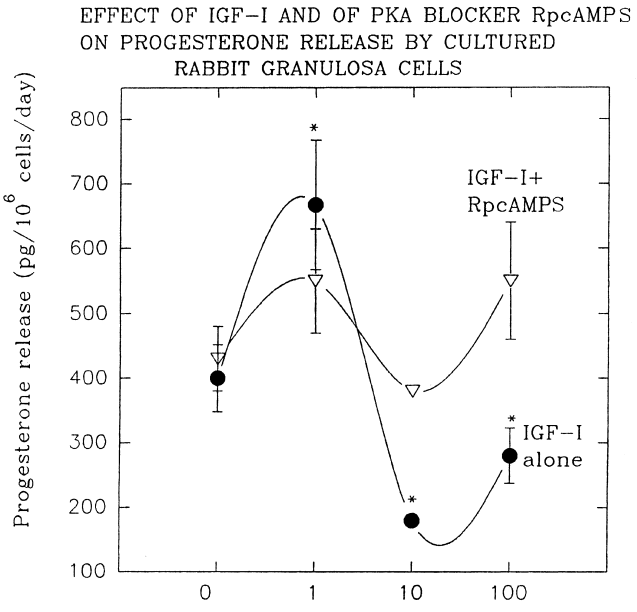

Fig. 1. Effects of IGF-I and Rp-cAMPS on progesterone release by cultured rabbit granulosa cells. ∗: Significant difference ($p < 0.05$) with control (without IGF-I added).

Table 2
Accumulation of IGF-I, progesterone, estradiol (at 72 h of culture), cAMP and cGMP (at 12 h of culture) in medium cultured with or without rabbit granulosa cells[a]

Substances measured	Unit of concentration in the medium	Medium	
		Cell-free	Cell-conditioned
IGF-I	ng/ml	0.89 + 0.17	11.23 + 0.65
Progesterone	pg/ml	13 + 4.7	320 + 38
Estradiol	pg/ml	ND[b]	91.3 + 9.53
cAMP	nM	ND[b]	3.87 + 1.22
cGMP	nM	ND[b]	0.2 + 0.06

[a] Isolated and washed granulosa cells were precultured in DME/F12 medium with 10% FCS for 2 days. The medium was then changed with fresh DME/F12 medium without FCS, and cells were cultured for a further 72 h (for hormone determination) or 12 h (for cyclic nucleotide determination). Simultaneously, culture medium incubated without cells was used as a blank control.
[b] Non-detectable.

3.2. Effect of IGF-I and protein kinase A blockade on the presence of proliferating cell nuclear antigen (PCNA) in cultured rabbit granulosa cells

Immunocytochemical analysis demonstrated PCNA-immunoreactivity (brown staining), localized to the nuclear region of cells (Fig. 5). IGF-I (100 ng/ml) more than doubled ($p < 0.001$) the proportion of PCNA-positive cells. Rp-cAMPS (50 nmol) given alone had no effect but in combination with IGF-I it partially prevented the IGF-I-induced increase in the proportion of PCNA-positive cells (Table 3).

3.3. Effect of IGF-I and protein kinase A blockade on the preimplantation development of rabbit zygotes in vitro

IGF-I (100 ng/ml medium) significantly ($p < 0.001$) increased the proportion of divided zygotes and of embryos reaching the morula/blastocyst stage. Neither blocker of PKA (Rp-cAMPS, 50 nmol or KT5720, 5 μg/ml) had a significant effect on embryo development when added alone but each was able to reverse the IGF-I effects on zygote cleavage and development (Table 4).

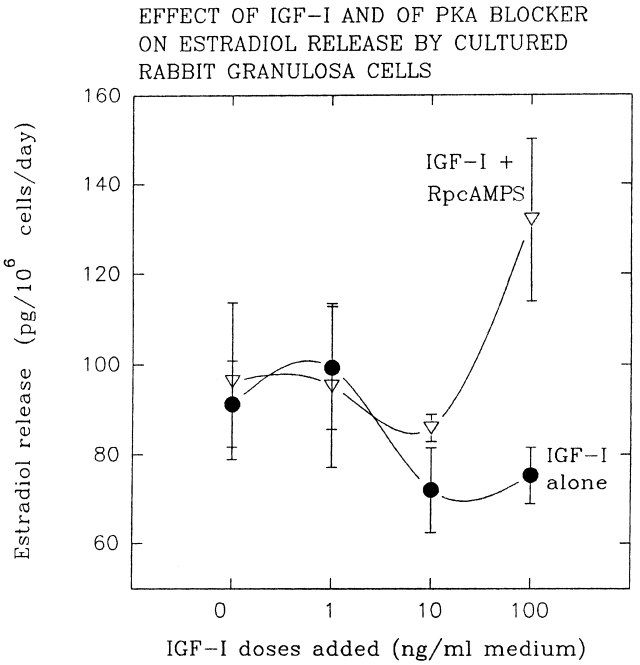

Fig. 2. Effects of IGF-I and Rp-cAMPS on estradiol release by cultured rabbit granulosa cells. *: Significant difference ($p < 0.05$) with control (without IGF-I added).

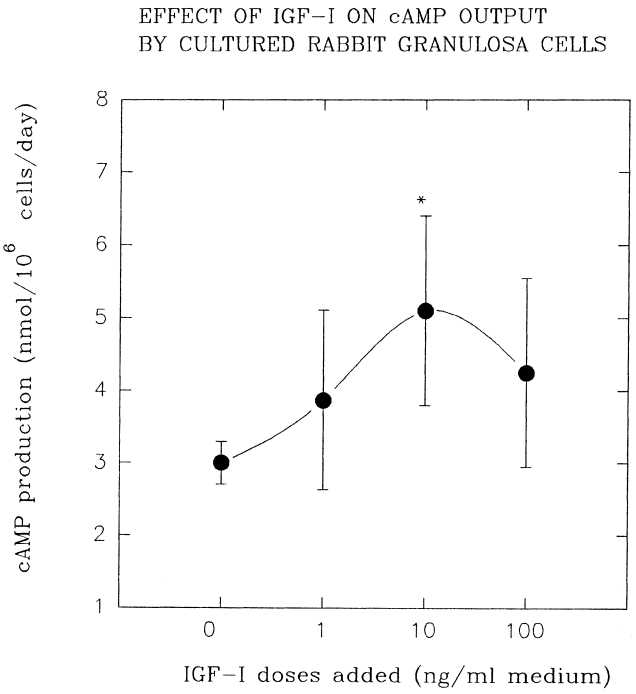

Fig. 3. Effect of IGF-I on cAMP secretion by cultured rabbit granulosa cells. *: Significant difference ($p < 0.05$) with control (without IGF-I added).

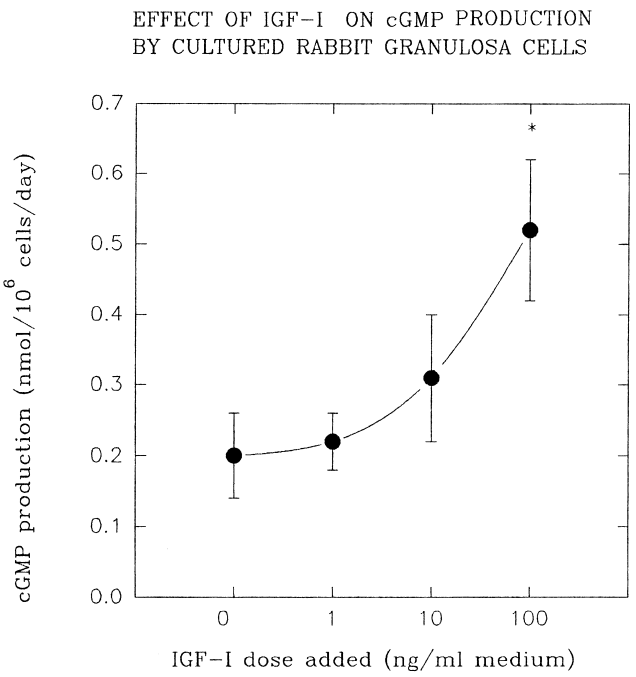

Fig. 4. Effect of IGF-I on cGMP secretion by cultured rabbit granulosa cells. ∗: Significant difference ($p < 0.05$) with control (without IGF-I added).

3.4. Effect of IGF-I and protein kinase A blockade on the presence of MAP- (ERKs) kinase in cultured rabbit granulosa cells

Immunocytochemical analysis of cells after culture demonstrated the presence of two different members of ERK family within the cells. ERK-1 immunoreactivity was distributed in the nuclear and perinuclear regions of the cells (Table 5, Fig. 6). ERK-3 immunoreactivity was localized exclusively in the cytoplasmic region as brown fibrous structures in the cytoskeleton (Fig. 7). Addition of IGF-I produced an almost 2.5-fold increase in the proportion of cells showing immunoreactivity to ERK-1 as well as to ERK3. Rp-cAMPS did not affect basal immunoreactivity to either ERK-1, or ERK-3 but it completely prevented any IGF-I-induced increase in the proportion of cells with immunoreactivity to ERK-3. The presence of immunoreactivity to active ERK-2 within rabbit granulosa cells

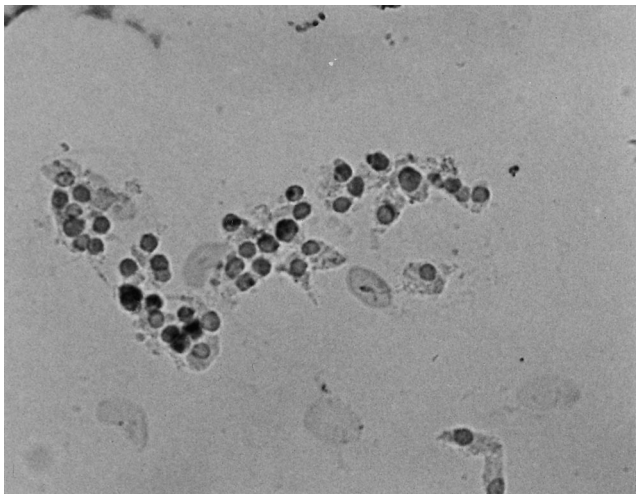

Fig. 6. ERK-1 MAP-kinase presence in cultured rabbit granulosa cells. (HRP-DAB detection, magnification ×400).

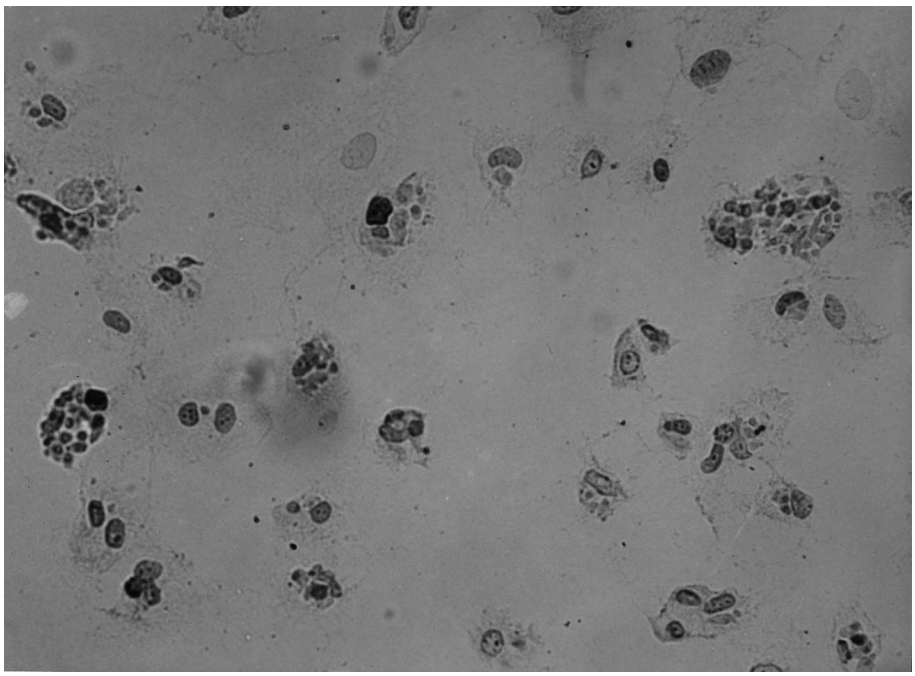

Fig. 5. PCNA presence in cultured rabbit granulosa cells. (HRP-DAB detection, magnification ×400).

Table 3
Effect of IGF-I and cAMP/PKA blocker on the presence of PCNA in cultured granulosa cells[a]

Treatment	Number of cells analyzed	PCNA-positive cells $X+m$ (%)
None (control)	3490	24.7+2.85
IGF-I (100 ng/ml)	3431	58.1+3.6[b]
Rp-cAMPS (50 nM)	3163	32.3+4.14
IGF-I + Rp-cAMPS	2998	38.2+2.4[b,c]

[a] Granulosa cells were isolated and precultured in chamber-slides for 2 days. Medium was then changed and the cells cultured for 16 h in fresh medium with treatments, washed in ice-cold PBS, fixed in ice-cold aceton and processed for the detection of PCNA.
[b] Significant difference with control at $p < 0.05$.
[c] Significant difference with IGF-I at $p < 0.05$.

Table 4
The effect of IGF-I, PKA-blockers and their combination on rabbit preimplantation embryo development[a]

Treatment groups	Number of treated embryos	Cleavage, (n/%)	Morula/Blastocyst (n/%)
None (control)	89	46/51.7	23/25.8
IGF-I (100 ng/ml)	108	92/85.2[b]	56/51.9[b]
Rp-cAMPS (50 nM)	77	39/50.6	26/33.7
IGF-I + Rp-cAMPS	58	67/63.7	18/31.0
KT5720 (5 μg/ml)	64	38/59.4	17/26.6
IGF-I + KT5720	60	32/53.3	14/23.3

[a] Zygotes were cultured in DME/F12 with treatments for 96 h. Stages of embryogenesis were evaluated according to the number and position of blastomeres. Presence of nuclei in blastomeres was detected by Giemsa staining according to Tarkowski [27].
[b] Significant difference with control at $p < 0.001$ (by Chi-square).

was determined by Western immunoblotting. This immunoreactivity was revealed in a 42 kDa band (the specificity was confirmed by comparison with an appropriate positive control). IGF-I (100 ng/ml) induced a visible increase in the intensity of this ERK-2-related band. Rp-cAMPS alone reduced band intensity below the control but it did not change the IGF-I-induced increase in the expression of the ERK-2-related band (Fig. 8).

4. Discussion

This study demonstrates that IGF-I can stimulate progesterone (but not estradiol), cAMP and cGMP release from cultured rabbit granulosa cells. It corresponds with previous reports that IGF-I is able to stimulate progesterone biosynthesis and production in cultured bovine [5], porcine [6] , ovine [7] granulosa and rabbit luteal [30] cells as well as in the rabbit intact perfused ovary [30]. The inability of IGF-I to affect estradiol release in our work, is in agreement with some previous reports of a lack of IGF-I influence on estradiol production by bovine ovarian cells [31], but does not correspond with other observations of stimulatory [9,10,13] or inhibitory [14,16] effects of IGF-I on estradiol secretion by bovine, porcine, human, murine, hamster and rabbit ovarian cells. These differences may be explained by the variety of materials studied (whole follicles, granulosa cells,

Table 5
Effect of IGF-I, Rp-cAMPS and their combination on the presence of ERKs in the cells[a]

Treatment	Number of cells analyzed	ERKs-positive cells (%, mean S.D.)	
		ERK-1	ERK-3
None (control)	3651	27.5+3.06	20.8+4.6
IGF-I (100 ng/ml)	3374	57.8+5.1[b]	51.3+7.5[b]
Rp-cAMPS (50 nM)	1680	24.6+6.8	21.6+4.9
IGF-I + Rp-cAMPS	2180	50.5+2.3[b]	23.4+7.7

[a] Granulosa cells were isolated and precultured in chamber-slides for 2 days. The medium was then changed and cells cultured for 20 min in a fresh medium with treatments, washed in ice-cold PBS and processed for the detection of ERK-1 and ERK-3.
[b] Significant difference with control at $p < 0.05$..

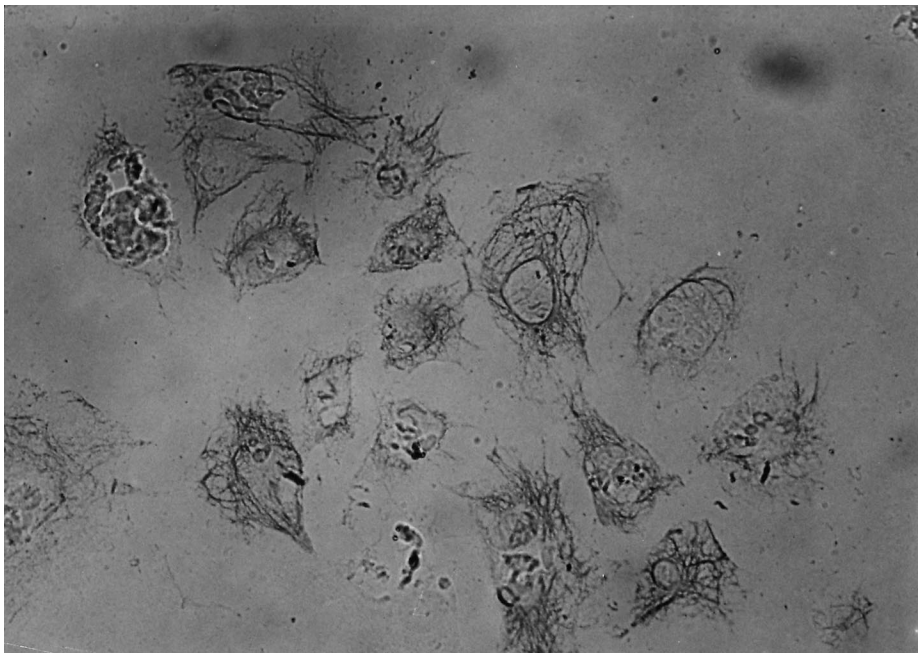

Fig. 7. ERK-3 MAP-kinase presence in cultured rabbit granulosa cells. (HRP-DAB detection, magnification ×400).

intact ovaries) and/or the experimental conditions (in vitro or in vivo). IGF-I reportedly stimulates estradiol but not progesterone release by perfused rabbit ovaries [10]. These data do not correspond with our observations of stimulatory effect of IGF-I on progesterone and a lack of IGF-I effect on estradiol release by rabbit granulosa cells. This discrepancy may be due to the spontaneous luteinization of granulosa cells in culture which is associated with a stimulation of progestagen and a suppression of estrogen secretions [2,3]. On the other hand, the relatively high estrogen accumulation (near 100 pg/10^6/day) in our experiment suggests that at least part of our granulosa cell culture was not luteinized and was producing large amounts of estradiol unaffected by IGF-I treatment.

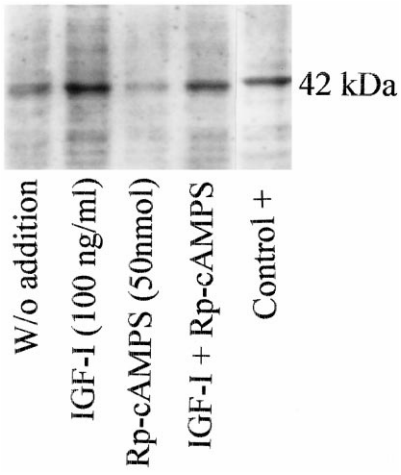

Fig. 8. Effects of IGF-I and Rp-cAMPS on the presence of active ERK-2 in lysates of rabbit granulosa cells. Granulosa cells were isolated and precultured for 2 days. The medium was then replaced with fresh serum-free medium containing treatments as indicated below, and the cells were cultured for 20 min, washed in ice-cold PBS, lysed with lysis buffer and subjected to Western-immunoblotting to visualize ERK-2. Treatments lane 1: medium without additions, 2: IGF-I (100 ng/ml), 3: Rp-cAMPS (50 nM), 4: IGF-I (100 ng/ml) in combination with Rp-cAMPS (50 nM), 5: human fibroblast cell lyzate (positive control).

The observation that IGF-I significantly stimulated proliferation (presence of PCNA) of granulosa cells is consistent with the well-known mitogenic properties of IGF-I on ovine [7] and bovine [5] granulosa cells. IGF-I also stimulated cleavage and increased the number of zygotes reaching the morula/blastocyst stage. This observation demonstrates that IGF-I has generative functions as well, and is consistent with previous reports of stimulation of embryogenesis in a number of species (mouse [17], buffalo [18], pig [19], cow [20], rabbit [21]). Moreover, Herrler et al. [21] found that IGF-I supports early embryonic development by preventing apoptosis and increasing cell proliferation, thereby suggesting an interrelation between early embryogenesis and proliferation. Looked at overall, our investigations indicate that IGF-I can be a stimulator of rabbit reproductive processes from ovarian steroidogenesis and proliferation to embryogenesis.

The ability of IGF-I to stimulate cyclic AMP release, observed in the present study, is consistent with previous observations on murine [25] and bovine [32] granulosa cells and on porcine ovarian follicles [33]. Since cAMP and cGMP are known intracellular

mediators of hormone action in various tissues including ovary [4], it is possible that cyclic nucleotides and related protein kinases are involved in mediating the IGF-I effect on ovarian tissue. This is supported by previous studies on bovine oviductal [34] and granulosa cells [35] which showed that IGF-I increases cAMP production and that PKA blockade prevents this effect. Our observation of a stimulatory influence of IGF-I on cAMP and of the prevention of IGF-I effects by PKA blockers suggests that the cyclic AMP/protein kinase A system may be involved in mediating the IGF-I action on secretory activity, cell proliferation and preimplantation zygote development. However, it is noted that IGF-I has a family of binding proteins (IGFBPs) which are believed to modulate its local action by regulating bioavailability [36]. It remains possible that the observed effects of IGF-I and regulators of cAMP/PKA may be modified or mediated by IGFBPs.

This appears to be the first report of an increase in cGMP production by ovarian cells after addition of IGF-I. Since cGMP can be elevated after activation of guanilyl cyclase by nitric oxide (NO) [37], it is possible that cGMP may be a mediator of IGF-I action on ovarian function. On the other hand, it is not known whether all the effects of NO are cGMP-dependent. Furthermore, NO can inhibit the production of progesterone [38,39] and estradiol [40] in rat and porcine ovarian cells, whereas in our study IGF-I stimulated progesterone and had no effect on estradiol production. Thus, no coincidence between the steroidogenic effects of NO and IGF-I is apparent. Moreover, it was demonstrated previously, that in porcine granulosa cells NO inhibits estradiol secretion independently of cGMP, (by inhibiting P450 aromatase activity) [40]. Taken together, these facts suggest indirectly that in our study IGF-I was not acting through NO. Confirmation of this conclusion must await studies with specific NO blockers.

The present results demonstrate the influence of the IGF-I/cAMP/PKA system on MAPK activity. They represent the first report of immunoreactive ERKs in rabbit granulosa cells. It is not known which member of the MAPK family is the main target of Rp-cAMPS action: given alone, this PKA blocker inhibited ERK-2 but not ERK-1 or ERK-3, whilst in combination with IGF-I it prevented ERK-3 but not ERK-1 or ERK-2 activation. It was reported previously, that MAPK can be activated by IGF-I in human granulosa cells [41] and by cAMP in porcine [42] and rat [43] granulosa cells. It is also reported that MAP kinase can mediate IGF-I effects on the proliferation of rat adipocytes [44] and the differentiation of human neuroblastoma cells [45], suggesting that it is involved in the IGF-I signal transduction pathway [46]. The reported attenuation of IGF-I-dependent activation of ERKs by cAMP in growth hormone-secreting granulosa cells [41] also confirms the interrelationship between the cAMP and ERK systems in mediating IGF-I actions.

The results of the immunocytochemical and Western blotting studies showed that IGF-I stimulated the activation of ERK-1, ERK-2 and ERK-3 members of MAPK family. They suggest that MAPK, like PKA, can be a potential mediator of IGF-I on ovarian cells. Furthermore, the effect of IGF-I on MAPK may be mediated via cAMP/PKA since in our experiments Rp-cAMPS was able to suppress the accumulation of MAPK and to prevent the stimulatory effect of IGF-I.

In conclusion, our in vitro study demonstrates that IGF-I is a potent stimulator of secretory, proliferative and generative functions in the female reproductive system of the rabbit. IGF-I effects are probably mediated by the cAMP/PKA system. ERK members of MAPK-family may be involved in the mediation of IGF-I. Furthermore, the influence of PKA blockers on ERKs suggests that IGF-I may regulate MAPK via the cAMP/PKA system.

Acknowledgements

The authors thank Mrs. T. Civanova, Mrs. K. Tothova, Ms. M. Kubekova and Ms. T. Gajarska for technical assistance with the isolation and analysis of rabbit zygotes and granulosa cells, as well as Dr. M. Luck (University of Nottingham) for editing the manuscript.

References

[1] J.F. Ackland, N.B. Schwartz, K.E. Mayo, R.E. Dodson, Nonsteroidal signals originating in the gonads, Physiol. Rew. 72 (1992) 731–787.
[2] R.J. Urban, J.D. Veldhuis, Endocrine control of steroidogenesis in granulosa cells. S.R. Milligan (Ed.), Oxford Rev. Reprod. Biol. 14 (1992) pp. 225–261.
[3] A.C. Paton, W.P. Collins, Differentiation processes of granulosa cells, Oxford Rev. Reprod. Biol. 14 (1992) 169–223.
[4] P. Leung, G. Steele, Intracellular signaling in the gonads, Endocr. Rev. 13 (1992) 476–498.
[5] D.T. Armstrong, P. Xia, G. de Gannes, F.R. Tekpetey, F. Khamsi, Differential effects of insulin-like growth factor-I and follicle-stimulating hormone on proliferation and differentiation of bovine cumulus cells and granulosa cells, Biol. Reprod. 54 (1996) 331–338.
[6] J.D. Veldhuis, J.T. Gwynne, Insulin-like growth factor type I (somatomedin-C) stimulates high density lipoprotein (HDL) metabolism and HDL-supported progesterone biosynthesis by swine granulosa cells in vitro, Endocrinology 124 (1989) 3069.
[7] D. Monniaux, C. Pisselet, Control of proliferation and differentiation of ovine granulosa cells by insulin-like growth factor-

I and follicle-stimulating hormone in vitro, Biol. Reprod. 46 (1992) 109–119.
[8] Z. Li, A.L. Johnson, Regulation of P450 cholesterol side-chain cleavage messenger ribonucleic acid expression and progesterone production in hen granulosa cells, Biol. Reprod. 49 (1993) 463–469.
[9] C.M. Foster, N. Hashimoto, J.F. Randolph, I.Z. Beitins, Comparison of growth hormone and insulin-like growth factor-I regulation of estradiol and progesterone production in human luteinized granulosa cells, Pediatric Res. 38 (1995) 763–767.
[10] Y. Yoshimura, M. Ando, S. Iwashita M. Nagamatsu, T. Adachi, K. Sueoka, T. Miyazaki, N. Kuji, M. Tanaka, 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.
[11] Y.P. Xu, P.J. Chedrese, P.A. Thacker, Growth hormone as an amplifier of insulin-like growth factor action: potentiated oestradiol accumulation, J. Endocrinol. 152 (1997) 201–209.
[12] E.Y. Adashi, C.E. Resnick, M.E. Svoboda, J.J. van Wyk, Somatomedin-C synergizes with follicle-stimulating hormone in the acquisition of progesterone biosynthetic capacity by cultured rat granulosa cells, Endocrinology 116 (1985) 2135–2142.
[13] S.Y. Chun, H. Billig, J.L. Tilly, I. Furuta, A. Tsafriri, A.J. Hsueh, Gonadotropin suppression of apoptosis in cultured preovulatory follicles: mediatory role of endogenous insulin-like growth factor-I, Endocrinology 135 (1994) 1845–1853.
[14] S. Roy, G.S. Greenwald, In vitro effects of epidermal growth factor, insulin-like growth factor-I, fibroblast growth factor, and follicle-stimulating hormone on hamster follicular deoxyribonucleic acid synthesis and steroidogenesis, Biol. Reprod. 44 (1991) 889–896.
[15] C.G. Gutierrez, B.K. Campbell, R. Webb, Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle-stimulating hormone, and morphological characteristics, Biol. Reprod. 56 (1997) 608–616.
[16] L.J. Spicer, E. Alpizar, R.K. Vernon, Insulin-like growth factor-I receptors in ovarian granulosa cells: effect of follicle size and hormones, Mol. Cell. Endocrinol. 102 (1994) 69–76.
[17] C. O'Neil, Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos in vitro, Biol. Reprod. 56 (1997) 229–237.
[18] A. Marula, M. Taneja, S.M. Totey, Morphological development, cell number, and allocation of cells to trophectoderm and inner cell mass of in vitro fertilized and parthenogenetically developed buffalo embryos: the effect of IGF-I, Mol. Reprod. Dev. 44, (1996) 343–351.
[19] P. Xia, F. Tekpetey, D. Armstrong, Effects of IGF-I on pig oocyte maturation, fertilization and early embryonic development in vitro, and on granulosa and cumulus cell biosynthetic activity, Mol. Reprod. Dev. 38 (1994) 373–379.
[20] G.A. Palma, G. Brem, Effect of high concentrations of IGF-I on the development of in vitro produced bovine embryos, Arch. Tierz. Dummerstorf 39 (1996) 85.
[21] A. Herrler, C.A. Krusche, H.M. Beier, Insulin and insulin-like growth factor-I promote rabbit blastocyst development and prevent apoptosis, Biol. Reprod. 59 (1998) 1302–1310.
[22] N. Costrici, G. Elberg, B. Lunenfeld, C. Pariente, J. Dor, H. Kanety, A. Karasik, A cytosolic protein tyrosine kinase activity is induced by follicle stimulating hormone and insulin like growth factor-I in human granulosa cells, Endocrinology 136 (1995) 4705–4708.
[23] F. Talavera, Z. Chen, K.M.J. Menon, IRS-I expression in the luteinized rat ovary: IGF-I and cyclic AMP effects on IRS-I tyrosine phosphorylation, Biochem. Biophys. Acta 1310 (1996) 10–18.
[24] J. Davis, J. May, B. Keel, Mechanisms of hormone and growth factor action in the bovine corpus luteum, Theriogenology 45 (1996) 1351–1380.
[25] E.Y. Adashi, C.E. Resnick, M.E. Svoboda, J.J. van Wyk, Somatomedin-C as an amplifier of FSH action: enhanced accumulation of adenosine 3′,5′-monophosphate, Endocrinology 118 (1986) 149–155.
[26] A.V. Sirotkin, A.V. Makarevich, Effect of growth factors on secretory functions of animal ovarian cells, Acta Zootechnica 52 (1996) 79–88.
[27] A.K. Tarkowski, An air-drying method for chromatin preparation for mouse eggs, Cytogenetics 5 (1966) 394–400.
[28] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 277 (1970) 680–685.
[29] M. Osborn, S. Isenberg, Immunohistochemistry of frozen and of paraffin tissue sections, in: J.E. Celis (Ed.), Cell Biology. A Laboratory Handbook, vol. II, Academic Press, New York, 1994, pp. 8, 361–367.
[30] S.H. Chen, V. Zanagnolo, S. Preutthipan, K.P. Roberts, S.B. Goodman, A.M. Dharmarajan, The role of insulin-like growth factor-I (IGF-I) and estradiol in rabbit corpus luteum progesterone production, Endocrine 6 (1997) 73–77.
[31] S.E. Echternkamp, L.J. Spicer, K.E. Gregory, S.F. Canning, J.M. Hammond, Concentrations of insulin-like growth factor-I in blood and ovarian follicular fluid of cattle selected for twins, Biol. Reprod. 43 (1990) 8–14.
[32] A.V. Sirotkin, A.V. Makarevich, The role of growth factors and tyrosine kinase in the control of apoptosis and secretory activity of bovine ovarian cells, J. Endocrinol. 156 (1998) 165.
[33] A.V. Sirotkin, A.V. Makarevich, J. Kotwica, P.-G. Marnet, H.B. Kwon, L. Hetenyi, Isolated porcine ovarian follicles as a model for the study of hormone and growth factor action on ovarian secretory activity, J. Endocrinol. 159 (1998) 313–321.
[34] A. Makarevich, A. Sirotkin, The involvement of the GH/IGF-I axis in the regulation of secretory activity by bovine oviduct epithelial cells, Anim. Reprod. Sci. 48 (1997) 197–207.
[35] P.-G. Marnet, A. Sirotkin, A. Makarevich, J. Bulla, Protein kinase A as a mediator of growth hormone and IGF-I effects on oxytocin release by bovine granulosa cells, J. Endocrinol. 152 (1997) 109.
[36] L.G. Giudice, Insulin-like growth factors and ovarian follicular development, Endocrine Rev. 13 (1992) 641–669.
[37] F. Murad, The role of nitric oxide in modulating guanylyl cyclase, Neurotransmissions (RBI) 10 (1994) 1–11.
[38] S. Ahsan, M. Lacey, S.A. Whitehead, Interactions between interleukin-1beta, nitric oxide and prostaglandin E_2 in the rat ovary: effects on steroidogenesis, Eur. J. Endocrinol. 137 (1997) 293–300.
[39] S. Dave, D.P. Farrance, S.A. Whitehead, Evidence that nitric oxide inhibits steroidogenesis in cultured rat granulosa cells, Clin. Sci. 92 (1997) 277–284.
[40] M. Masuda, T. Kubota, S. Kamada, T. Aso, Nitric oxide inhibits steroidogenesis in cultured porcine granulosa cells, Mol. Hum. Reprod. 3 (1997) 285–292.
[41] J. Webster, D. Prager, S. Melmed, Insulin-like growth factor-I activation of extracellular signal-related kinase-1 and -2 in growth hormone-secreting cells, Mol. Endocrinol. 8 (1994) 539–544.
[42] M.R. Cameron, J.S. Foster, A. Bukovsky, J. Wimalasena, Activation of mitogen-activated protein kinases by gonadotropins and cyclic adenosine 5′-monophosphates in porcine granulosa cells, Biol. Reprod. 55 (1996) 111–119.
[43] S. Das, E.T. Maizels, D. DeManno, E. St. Clair, S.A. Adam, M. Hunzicker-Dunn, A stimulatory role of cyclic adenosine 3′,5′-monophosphate in follicle-stimulating hormone-activated

mitogen-activated protein kinase signalling pathway in rat ovarian granulosa cells, Endocrinology 137 (1996) 967–974.
[44] A. Porras, A.M. Alvarez, A. Valladares, M. Benito, p42/p44 mitogen-activated protein kinases activation is required for the insulin-like growth factor-I/insulin induced proliferation, but inhibits differentiation, in rat fetal brown adipocytes, Mol. Endocrinol. 12 (1998) 825–834.
[45] B. Kim, P.S. Leventhal, A.R. Saltiel, E.L. Feldman, Insulin-like growth factor-I-mediated neurite outgrowth in vitro requires mitogen-activated protein kinase activation, J. Biol. Chem. 272 (1997) 21,268–21,273.
[46] R. Foncea, M. Anderson, A. Ketterman, V. Blakesley, M. Sapag-Hagar, P.H. Sugden, D. LeRoith, S. Lavandero, Insulin-like growth factor-I rapidly activates multiple signal transduction pathway in cultured rat cardiac myocytes, J. Biol. Chem. 272 (1997) 19,115–19,124.