PL-ISSN 0015-5497 (print), ISSN 1734-9168 (online)
Folia Biologica (Kraków), vol. 62 (2014), No 3

doi:10.3409/fb62_3.211

Short communication

The cAMP Analogue, dbcAMP, Affects Rabbit Ovarian Cell Proliferation, Apoptosis, Release of Steroids and Response to Hormones*

Alexander V. SIROTKIN, Andrej BALAZI, and Peter CHRENEK

Accepted May 15, 2014

SIROTKIN A. V., BALAZI A., CHRENEK P. 2014. The cAMP analogue, dbcAMP, affects rabbit ovarian cell proliferation, apoptosis, release of steroids and response to hormones. Folia Biologica (Kraków) **62**: 211-218.

Cyclic adenosine monophosphate (cAMP)-related intracellular mechanisms can be important in the control of reproductive processes. The aim of the present study was to examine possible hormonal and intracellular mechanisms mediating the action of cAMP on ovarian cell functions, in particular the mechanisms of stimulatory action of dbcAMP on rabx and caspase 3), plasma level of progesterone and estradiol, and release of progesterone, testosterone and estradiol by isolated ovarian fragments, as well as the response of these fragments to hormonal regulators of ovarian steroidogenesis – FSH, IGF-I and ghrelin (all at doses of 100 ng/ml). Release of hormones was assessed by RIA. The expression of markers of proliferation and apoptosis was evaluated by electrophoresis-Western blotting. Administration of dbcAMP resulted in a reduction of the estradiol level, but not progesterone level in rabbit plasma, as well as in progesterone and estradiol release, but not testosterone release, by isolated ovarian fragments. Additions of FSH, IGF-I and ghrelin to culture medium reduced the release of progesterone and increased testosterone and estradiol output by fragments isolated from control animals. The release of all these hormones was increased by fragments isolated from dbcAMP-treated animals. Both dbcAMP injections and addition of FSH, IGF-I and ghrelin to culture medium promoted the accumulation of proliferation and apoptosis markers in cultured ovarian fragments, whilst dbcAMP increased the stimulatory effects of hormones on these markers. The present observations (1) confirm involvement of peptide hormones FSH, IGF-I and ghrelin in the control of ovarian cell proliferation, apoptosis and steroid hormone release; (2) demonstrate the involvement of cAMP-dependent intracellular mechanisms in promotion of rabbit ovarian cell proliferation, apoptosis and in regulation of ovarian steroidogenesis; (3) suggest the involvement of cAMP-dependent mechanisms in mediating and/or promoting the effect of peptide hormones FSH, IGF-I and ghrelin on ovarian cell proliferation, apoptosis, but not on steroidogenesis; (4) suggest that the stimulatory action of dbcAMP on rabbit reproduction could be due to dbcAMP-induced changes in ovarian cell proliferation, apoptosis, steroid hormone release and the response of these processes to hormonal regulators.

Key words: dbcAMP, rabbit, ovary, progesterone, testosterone, estradiol, proliferation (PCNA), apoptosis (bax).

Alexander V. *SIROTKIN, Institute of Genetics and Animal Reproduction, Research Institute of Animal Production Centre Nitra, 951 41 Lužianky near Nitra, Slovak Republic; Department of Zoology and Anthropology, Constantine the Philosopher University, 949 74 Nitra, Slovak Republic. E-mail: sirotkin@cvzv.sk*
Andrej BALAZI, Institute of Genetics and Animal Reproduction, Research Institute of Animal Production Centre Nitra, 951 41 Lužianky near Nitra, Slovak Republic.
Peter CHRENEK, Institute of Genetics and Animal Reproduction, Research Institute of Animal Production Centre Nitra, 951 41 Lužianky near Nitra, Slovak Republic; Department of Biochemistry and Biotechnology, Slovak University of Agriculture in Nitra, 949 72 Nitra, Slovak Republic.

The study and application of intracellular regulators of reproduction may be important for the control of animal and human fecundity. Cyclic nucleotide adenosine monophosphate (cAMP) can be involved in the control of reproductive processes. It was shown that pharmacological regula-

*Supported by the Ministry of Agriculture of Slovak Republic (project MP SR 01).

tors of cAMP and its effector, cAMP-dependent protein kinase A (PKA), can control ovarian cell proliferation (PELUSO *et al*. 1993; GRAWES & LAWRENCE 1996; HSUEH 1996; HILLIER & TETSUKA 1997; CHEADLE *et al*. 2008; VIEGAS *et al*. 2008; SIROTKIN *et al*. 2010a), apoptosis (VIEGAS *et al*. 2008; SIROTKIN *et al*. 2010a; SPACZYNSKI *et al*. 2005; AMSTERDAM *et al.* 2003) and ovarian follicle development (FRANKS *et al*. 2000). The involvement of cAMP/PKA in control of ovarian functions can be practically used for control of reproductive processes. For example, synthetic inhibitors of cAMP-specific phosphodiesterase (PDE, cyclic nucleotide degrading enzyme) increased cAMP accumulation in ovarian cells, as well as the number of ovulations, embryos and born pups in gonadotropin-stimulated rats (MCKENNA *et al.* 2005). The cAMP- and cGMP-specific PDE inhibitor, 3-isobutyl-methyl-xanthine (IBMX) stimulated rabbit ovarian folliculogenesis, affected ovarian and oviduct cell proliferation, apoptosis and release of hormones (SIROTKIN *et al*. 2010a) and increased ovarian luteinization, number of ovulations and embryo yield in rabbits (SIROTKIN *et al*. 2010 a; SIROTKIN *et al*. 2008a). Similar effects on rabbit reproduction (increase in number of ovulations, *corpora lutea*, oocyte and embryo yield) was achieved by a more specific pharmacological activator of cAMP-dependent intracellular mechanism – synthetic cAMP cell-permeable analogue, N^6,2'-dibutyryladenosine 3'5'-cyclic monophosphate (dbcAMP) by CHRENEK *et al.* (2012). dbcAMP was shown to stimulate the release of rabbit ovarian hormones (CHRENEK *et al.* 2010). These findings indicate that activators of cAMP--dependent signalling pathways, including dbcAMP, may promote ovarian functions.

The mechanisms of action of cAMP regulators on ovarian functions have been studied insufficiently. One such mechanism may include reproductive hormones. Involvement of FSH, IGF-I, ghrelin, steroid and other hormones in control of ovarian functions in rabbits (CHRENEK *et al.* 2010; SIROTKIN *et al*. 2008b; SIROTKIN *et al*. 2009) and in other mammals (HILLIER 2001; SIROTKIN 2005; PELUSO 2006) are well-known. First, cAMP can affect hormonal regulators of reproduction: cAMP is a potent promoter of GnRH (POON *et al.* 2008), whilst pharmacological regulators of cAMP are able either to promote or to suppress the release of steroid and peptide hormones by ovarian cells *in vitro* (HILLIER & TETSUKA 1997; SIROTKIN *et al*. 2010a; AMSTERDAM *et al.* 2003; SIROTKIN 2005; PELUSO 2006; SCHAEFFER & SIROTKIN 1995; MAKAREVICH *et al.* 2004a; PIERRE *et al*. 2004). Second, cAMP can affect hormone reception. Analogues of cAMP can either decrease (MURPHY & DOBIAS 1999) or increase (NAKAMURA *et al*. 1995; TANO *et al*. 1997) the expression of FSH receptors and its mRNA, whilst PDE inhibitor can reduce expression of LH receptors (PEEGEL *et al*. 2005). Third, cAMP can be a post-receptor mediator of action of hormones promoting ovarian functions and, therefore, either enhance or suppress the action of these hormones on the ovary. Pharmacological regulators of cAMP/PKA can modify the response of ovarian cells to LH-RH (SIROTKIN *et al*. 1994; RAMAKRISHNAPPA *et al*. 2005), gonadotropins (HILLIER & TETSUKA 1997; MCKENNA *et al.* 2005; CHRENEK *et al*. 2010; HILLIER 2001; POON *et al.* 2008; SIROTKIN *et al*. 1994; HUA *et al*. 2008), GH (SIROTKIN 2005; MAKAREVICH & SIROTKIN 2000), IGF-I (CHRENEK *et al*. 2010; MAKAREVICH & SIROTKIN 2000), oxytocin (SIROTKIN 1996; MAKAREVICH *et al.* 2004b), ghrelin (CHRENEK *et al*. 2010), bone morphogenetic protein-4 (PIERRE *et al*. 2004) and progestins (PELUSO 2006). Thus, pharmacological activation of the cAMP/PKA system could alter either the release or reception and action of the key hormonal regulators of reproductive processes.

Although regulators of cAMP/PKA are able to affect the hormonal stimulators of reproduction listed above and their effects, the available knowledge concerning the direct action of cAMP on ovarian hormones is insufficient and contradictory. Some authors observed stimulatory action of cAMP agonists and inhibitory action of cAMP antagonists on the release of progesterone, estradiol and IGF-I by porcine ovarian cells (MAKAREVICH *et al.* 2004a; MAKAREVICH & SIROTKIN 2000) and a stimulatory action of a cAMP agonist on estradiol release by rat granulosa cells (PELUSO *et al*. 1993). On the contrary, some authors (PIERRE *et al*. 2004) reported that both PDE inhibitor and dbcAMP suppress the release of ovarian progesterone in sheep granulosa cells, whilst other studies failed to demonstrate any action of cAMP/PKA blocker on progesterone release by rabbit granulosa cells (MAKAREVICH *et al.* 2000) or inhibin secretion by mouse ovaries (HUA *et al*. 2008). As concerns cAMP action on hormone reception and action, the effects of regulators of cAMP on action of gonadotropins and IGF-I have been insufficiently studied and their influence on ghrelin action on mammalian ovaries was not examined yet. The ability of cAMP regulators to modify the action of hormones on rabbit ovaries has not been investigated at all.

Besides hormones, cAMP/PKA can control reproductive functions affecting proliferation and/or apoptosis of ovarian cells. There is evidence that cAMP/PKA can either up- (SPACZYNSKI *et al*. 2005; AMSTERDAM *et al.* 2003; SIROTKIN *et al*. 2010b) or down (VIEGAS *et al*. 2008) – regulate rat, human and rabbit ovarian cell apoptosis. Moreover, cAMP/PKA was shown to be either an

inhibitor (PELUSO *et al*. 1993; GRAWES & LAWRENCE 1996; HSUEH *et al*. 1996; HILLIER & TETSUKA 1997) or stimulator (CHEADLE *et al*. 2008; VIEGAS *et al*. 2008; SIROTKIN *et al*. 2010b) of proliferation of these cells. In our recent experiments on rabbits (CHRENEK *et al.* 2012), dbcAMP administration resulted in an increase of ovarian follicular atresia, but increased ovarian mass, indicating that cAMP/PKA can stimulate both ovarian cell apoptosis and proliferation in this species. This hypothesis requires direct confirmation because the influence of pharmacological regulators of cAMP on markers of proliferation and apoptosis in rabbit ovaries has not been studied yet.

Therefore, the available information concerning the action of cAMP regulators on release of steroid hormones, their ability to modify effects of hormonal regulators of ovarian functions, as well as on ovarian cell proliferation and apoptosis remain insufficient, inconsistent and contradictory. In addition, previous data concerning the role of cAMP-dependent intracellular mechanisms in control of reproduction were obtained mainly in *in-vitro* experiments, therefore they require confirmation by *in-vivo* data. On the other hand, the few results obtained in previous *in-vivo* experiments (CHRENEK *et al*. 2012) provide no evidence on mechanisms of cAMP action.

The aim of our present study was to examine possible hormonal and intracellular mechanisms mediating the action of cAMP/PKA on ovarian cell functions, in particular the mechanisms of stimulatory action of dbcAMP on rabbit ovarian growth, folliculogenesis, and oocyte and embryo production observed previously (CHRENEK *et al*. 2012). For this purpose, we examined the influence of administration of dbcAMP, a cAMP analogue, on basic ovarian functions in rabbits – proliferation (expression of proliferation-related antigen, PCNA) (MAGA & HUBSCHER 2003), apoptosis (expression of apoptosis-related proteins, bax and caspase 3) (HARADA & GRANT 2003; DEGLI ESPOSTI 2004), plasma level of steroid hormones (progesterone and estradiol), release of steroid hormones (progesterone, testosterone and estradiol) by isolated ovarian fragments, as well the response of these fragments to some known hormonal regulators of ovarian steroidogenesis – FSH, IGF-I and ghrelin.

Material and Methods

Manipulations with animals

The induction of the ovarian cycle by using gonadotropins and gonadotroiously (CHRENEK *et al.* 2012). Briefly, female rabbits 4 months of age were kept in cages under standard conditions in Animal Production Research Centre, Nitra, Slovak Republic, were treated three days before mating with pregnant mare serum gonadotropin (PMSG, Werfaser, Alvetra und Werfft AG., Vienna, Austria, 100 IU/animal) followed after 72 h by human chorionic gonadotropin (hCG, Werfachor, Alvetra und Werfft AG, 200 IU/animal). Control animals were i.m injected only with these gonadotropins (in 0.7 ml saline per animal), whilst experimental females received gonadotropins together with dbcAMP (Biolog Life Science Institute, Bremen, Germany, 5 or 50 μg/animal, all in 0.7 ml saline per animal). All substances were injected intramuscularly. At 19-20 h after insemination, all females were killed by decapitation, blood was collected, centrifuged at 400xg, and the separated plasma frozen at -18^{o}C to await RIA. The ovaries were weighed and collected for subsequent culture. All experiments were carried out with the approval of a local ethical commission in accordance to Slovak and EU regulations concerning animal experiments. Concentrations of both progesterone and estradiol in plasma samples collected from rabbits treated with dbcAMP at doses 5 or 50 μg/animal was analysed. The ability of dbcAMP to stimulate rabbit reproductive efficiency when administrated at this but not at lower doses was demonstrated previously CHRENEK *et al*. 2012). The plasma level of progesterone and estradiol, as well as the release of progesterone, testosterone and estradiol by ovarian fragments isolated from rabbits treated and not treated with dbcAMP and cultured with and without exogenous hormones (see below) were examined by RIA. Expression of markers of both proliferation and apoptosis in these fragments were evaluated by Western blotting.

Preparation, culture and processing of ovarian cells

Ovaries were processed and cultured as described previously (SIROTKIN *et al*. 2008b). Briefly, ovaries were washed in PBS with 1% antibiotic-antimycotic solution (Sigma, St. Louis, USA), placed in 100 mm diameter culture dishes (Gama, České Budejovice, Czech Republic) and dissected using a blade knife, and the resulting 1/8 of ovaries (3-4 mg pieces) were washed 3 times in sterile PBS. Fragments of ovaries were cultured in Falcon 24-well plates (Becton Dickinson, Lincoln Park, USA, 2 randomly selected fragments/well/2ml medium) in sterile culture medium (DMEM/F-12 1:1 mixture supplemented with 10% bovine fetal serum and 1% antibiotic-antimycotic solution, all from Sigma) at 37^{o}C and 5% CO_2in humidified air. Ovarian fragments in both control animals and animals treated with dbcAMP (50 ìg/animal) were

cultured with and without pFSH (Sigma), recombinant human IGF-I (Sigma) and human recombinant ghrelin 1-18 (Peptides International Inc., Louisville, Kentucky, USA). All hormones were of research grade, all were dissolved in incubation medium immediately before experiments at concentration 100 ng/ml medium each. No pre-culture of fragments without hormones was performed. After 2 days of culture, the medium from the plate wells was aspirated and frozen at -18°C to await RIA. The ovarian fragments were lysed by 3 cycles of freezing (at -70°C) – thawing (at room temperature) and vigorous pipetting in ice-cold electrophoretic buffer (0.0625M Tris-base, 2% SDS, 10% glycerol, 0.5% 2-mercaptoethanol, 0.003% bromophenol blue, all from Sigma, 25 μl/sample). The supernatant was separated from cellular membranes by centrifugation for 10 min at 200 x g and frozen at -18°C to await electrophoresis and Western immunoblotting.

Immunoassay

Concentrations of progesterone, testosterone and estradiol were determined in 25 of incubation medium and blood plasma by RIA kits for progesterone and estradiol from DSL (Webster, Texas, USA) and RIA kit for testosterone from Immunotech (Marseille, France) according to the manufacturer's instructions. The RIAs were validated for use in samples of culture medium.

The applied progesterone antiserum cross-reacted less than 0.001% to cortisol, corticosterone, androstenediol, pregnenolone, estradiol and testosterone. Sensitivity of the assay was 0.12 ng/ml, intra- and inter-assay coefficients of variation did not exceed 13.1% and 8.0%, respectively.

Testosterone antiserum had less than 0.001% cross-reactivity with DHEA, progesterone, estradiol and other related molecules. Sensitivity of the assay was 0.025 ng/ml, intra- and interassay coefficients of variation were below or equal to 14.8% and 15.0%, respectively.

The cross-reactivity of antiserum against estradiol was less than 0.01% to DHEA, progesterone, cortisol, androsterone, testosterone, corticosterone and cortisone. Sensitivity of the E_2 was 6.5 ng/ml, maximal intra- and inter-assay coefficients were 19.2% and 9.4%, respectively.

Protein gel electrophoresis and Western immunoblotting

Lysates of ovarian tissue were mixed 1:1 with electrophoretic buffer (0.0625M Tris-base, 2% SDS, 10% glycerol, 0.5% 2-mercaptoethanol, 0.003% bromophenol blue; all from Sigma), boiled at 95°C for 3 min and subjected to SDS-polyacrylamide gel electrophoresis in 4% and 10% stacking and resolving gels, respectively, at 25 mA constant current according to Laemmli (1970). The samples were then transferred to Porablot PVDF membranes (Macherey-Nagel, Duren, Germany) using a semi-dry trans-blotter (Bio-Rad Labs, Richmond, USA). Endogenous peroxidase in samples was quenched by incubation in 3% H_2O_2 for 15 min. Non-specific binding of antiserum was prevented by incubation in 5% blot-qualified BSA (Amersham plc, Little Clafton, UK) in TTBS (20 nM Tris-base, 137 nM NaCl, 0.1% Tween-20). Blocked membranes were probed with mouse monoclonal antibodies against PCNA, caspase 3, bax and housekeeping protein GAPDH (binds corresponding antigens of human, mouse and rat origin; dilution 1:250; all from Santa Cruz Biotechnology, Inc., Santa Cruz, USA). Membranes were then incubated with secondary horseradish peroxidase-conjugated porcine anti-mouse IgG antibody (Sevac, Prague, Czech Republic) and visualized using Super-Signal West-Pico luminescent substrate (Pierce, Rockford, Il, USA) and ECL Hyper-film (Amersham) according to the instructions of the manufacturer. Incubation medium without cells was used as a negative (blank) control. Molecular weights of fractions were evaluated using a molecular weight calibration kit (18, 24, 45 and 67 kD; ICN Biomedicals Inc, Irvine, USA).

Statistics

Each experiment was performed on 6 control animals and 6 animals treated with dbcAMP. Animals from each group were killed to collect blood and ovaries for subsequent analysis. Each experiment was performed 6 times. Each *in vitro* experimental group was represented by four culture wells. The data shown are means of values obtained in 4 separate experiments using different animals and their ovaries. After RIA, the values of blank controls (medium incubated without cells) were subtracted from the specific values determined in cell-conditioned medium to exclude any non-specific background (less than 15% of total values). The rates of substance secretion were calculated per mg tissue/day. The samples intended for RIA were analysed separately, whilst the samples intended for SDS PAGE-Western blotting (total 6 samples/treatment obtained in 6 experiments) were pooled before processing. Significant differences between the treatments were evaluated by using two-way ANOVA followed by paired t-tests or by Duncan's tests (Sigma Plot 11.0 statistical software, Systat Software, GmbH, Erkrath, Germany). Differences from control at $P<0.05$ were considered as significant.

Results

RIA detected the presence of both progesterone and estradiol in rabbit blood plasma (Fig. 1), as well as the accumulation of progesterone, testosterone and estradiol in medium conditioned by cultured ovarian fragments (Fig. 2). Electrophoresis-Western immunoblotting demonstrated the presence of proliferation-related substance PCNA and apoptosis-related substances caspase 3 and bax in cultured ovarian tissue. PCNA and caspase 3 were represented by two fractions of similar molecular weight (Fig. 3).

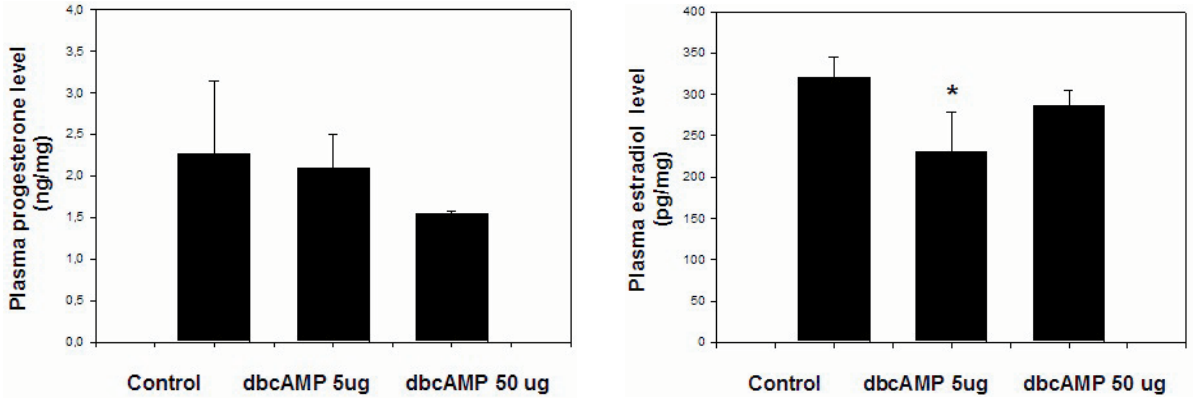

Fig. 1. Effect of dbcAMP injections (5μg/animal and 50 μg /animal) on the plasma level of (a) progesterone and (b) estradiol in rabbit plasma. RIA data. Values are means$\pm$S.E.M., * – significant ($P<0.05$) differences with control (no dbcAMP treatment).

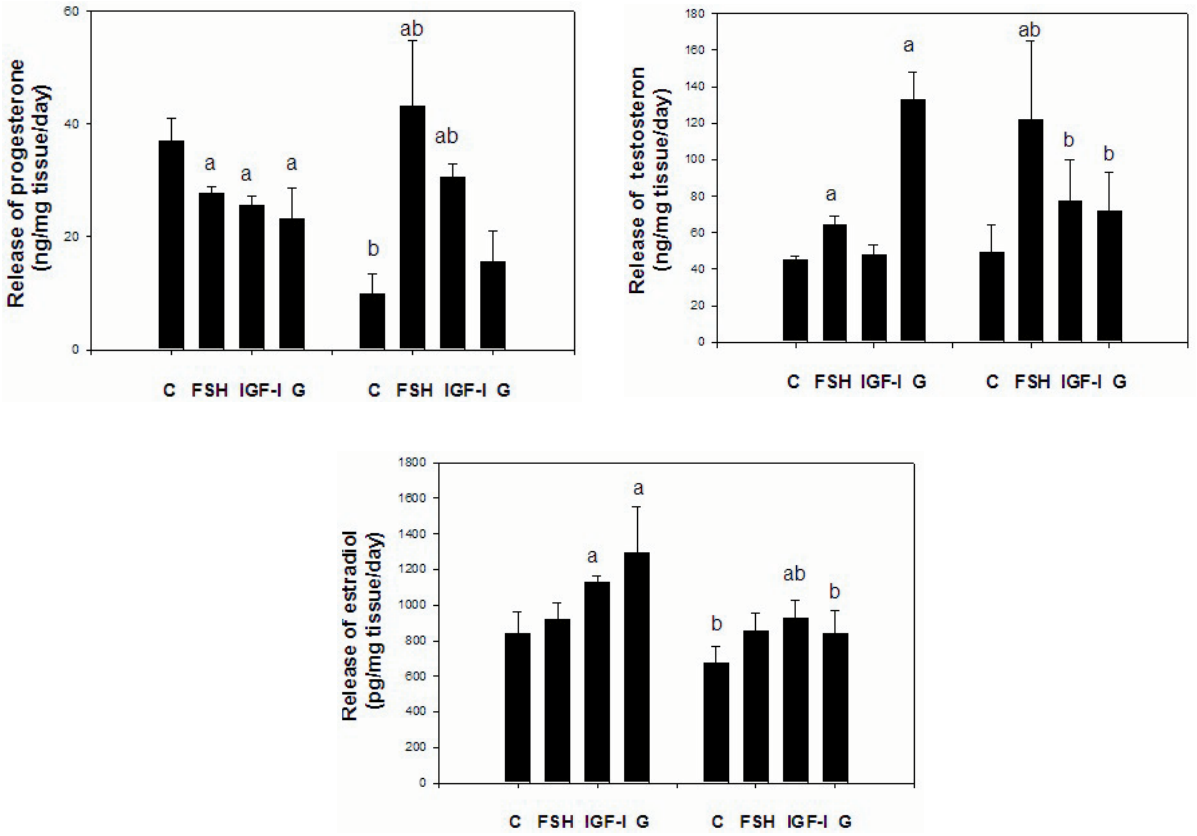

Fig. 2. Effect of dbcAMP (50 μg/animal) injections *in vivo* on the subsequent ability of rabbit ovarian fragments cultured with and without FSH, IGF-I and ghrelin (all at 100 ng/ml medium) to release (a) progesterone, (b) testosterone and (c) estradiol *in vitro*. RIA data. Left – fragments of ovaries isolated from control rabbits, right – fragments of ovaries isolated from rabbits treated with dbcAMP. Values are means$\pm$S.E.M., a – effect of hormone addition: significant ($P<0.05$) differences between ovarian fragments cultured with and without hormones, b – effect of administration of dbcAMP: significant ($P<0.05$) differences between corresponding groups of fragments of ovaries isolated from rabbits treated and not treated with dbcAMP.

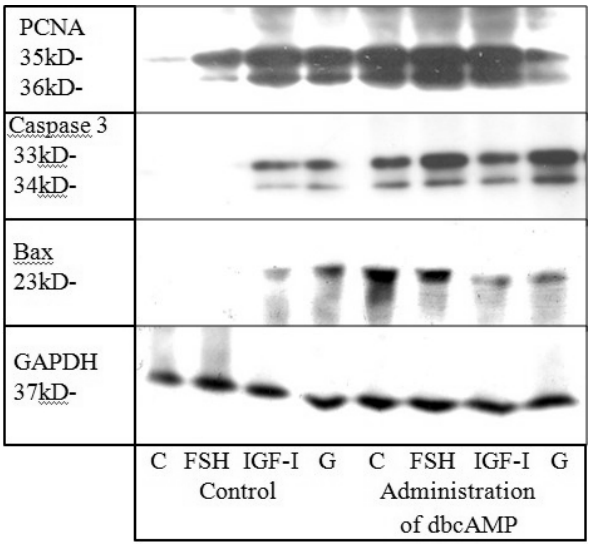

Fig. 3. Effect of dbcAMP injections (50 μg/animal) *in vivo* on the subsequent expression of PCNA, caspase 3, bax and housekeeping protein GAPDH in rabbit ovarian fragments cultured with and without FSH, IGF-I and ghrelin (all at 100 ng/ml medium). Data from electophoresis-Western immunoblotting. Molecular weights are indicated in kilodaltons (kD).

A comparison of hormone level in blood plasma of rabbits treated and not treated with dbcAMP (at dose 50 μg/animal) did not show a substantial difference in progesterone level (Fig. 1a). Nevertheless, administration of dbcAMP resulted in a significant decrease in plasma estradiol level (Fig. 1b).

Tissue isolated from ovaries of rabbits treated by dbcAMP (50 μg/animal) and cultured without exogenous hormones released significantly less progesterone than tissue from rabbits not treated with dbcAMP. Addition of FSH, IGF-I or ghrelin to the culture medium significantly reduced the progesterone output by ovarian fragments of control rabbits. In ovarian tissue isolated from animals treated with dbcAMP, FSH or IGF-I, but not ghrelin, stimulated progesterone release (Fig. 2a).

No significant differences in basal testosterone release between ovarian tissue isolated from control and dbcAMP-treated rabbits were found. In ovarian fragments isolated from control animals, the addition of FSH and ghrelin, but not IGF-I, significantly promoted testosterone output. Testosterone release was stimulated by additions of all tested hormones in ovarian fragments of dbcAMP-treated animals (Fig. 2b).

Basal estradiol release was significantly lower in ovarian tissue isolated from dbcAMP-treated animals than from control rabbits. In both groups estradiol release was significantly increased under the influence of additions of IGF-I and ghrelin, but not of FSH (Fig. 2c).

Accumulation of PCNA in ovarian tissue isolated from rabbits treated with dbcAMP was much higher than in control animals. Addition of FSH, IGF-I and ghrelin to culture medium increased accumulation of this proliferation-related peptide in ovaries of control animals. In cultured ovarian tissue of dbcAMP-treated rabbits, FSH and IGF-I did not affect PCNA accumulation, and addition of ghrelin reduced it.

Ovaries isolated from dbcAMP-treated rabbits were characterized by much higher accumulation of caspase 3 than ovaries of control animals. Addition of hormones to culture medium increased expression of caspase 3 in ovarian fragments isolated from either the control group (caspase increased after addition of IGF-I and ghrelin) and from dbcAMP-treated group (caspase increased after addition of FSH and ghrelin).

The administration of dbcAMP also resulted in a substantial increase in accumulation of bax. In control ovaries, this index also increased after the addition of IGF-I and ghrelin. In the dbcAMP-treated group, addition of each hormone induced a decrease in bax accumulation (Fig. 3).

Discussion

The presence of steroid hormones in rabbit blood, their release by cultured ovarian tissue, as well as the expression of proliferation- and apoptosis-related substances in rabbit ovaries are in line with our previous observations (SIROTKIN *et al*. 2010b; CHRENEK *et al.* 2010; SIROTKIN *et al*. 2008b; SIROTKIN *et al*. 2009; MAKAREVICH *et al.* 2000).

The visible increase in PCNA accumulation observed in dbcAMP-treated rabbit ovarian cells (Fig. 3) is the first evidence that dbcAMP is a stimulator of rabbit ovarian cell proliferation. This observation is in agreement with previous reports on stimulating (CHEADLE *et al*. 2008; VIEGAS *et al*. 2008; SIROTKIN *et al*. 2010b) but not on inhibiting (PELUSO *et al*. 1993; GRAWES & LAWRENCE 1996; HSUEH *et al*. 1996; HILLIER & TETSUKA 1997) action of cAMP/PKA on proliferation of ovarian cells in other species. These observations are in line with previous reports on involvement of cAMP-dependent intracellular mechanisms in up-regulation of ovarian follicular growth in rat (MCKENNA *et al.* 2005) and rabbit (SIROTKIN *et al*. 2008a; CHRENEK *et al*. 2012; SIROTKIN *et al*. 2010c). Nevertheless, this is the first evidence that cAMP-induced rabbit ovarian follicular growth can be due to promotion of cell proliferation. It is less probable that cAMP-induced stimulation of ovarian functions is due to a decrease in apoptosis because the administration of dbcAMP did not inhibit, but rather stimulated the accumulation of cytoplasmic apoptosis markers caspase 3 and bax.

Our observations correspond to reports on the ability of cAMP/PKA to stimulate ovarian cell apoptosis (SPACZYNSKI *et al*. 2005; AMSTERDAM *et al.* 2003; SIROTKIN *et al*. 2010b), but not to its anti-apoptotic role (VIEGAS *et al*. 2008) in ovaries in other species. This is the first data on the action of dbcAMP on rabbit ovarian apoptosis. Our observations correspond to the increase in morphological signs of atresia observed in rabbit ovaries after administration of PDE inhibitor (SIROTKIN *et al*. 2010c) or dbcAMP (CHRENEK *et al*. 2012). The dbcAMP-induced accumulation of both proliferation- and apoptosis-related substances suggest that dbcAMP administration can promote cellular turnover (replacement) in rabbit ovaries.

Administration of dbcAMP was able to reduce the release of steroid hormones by rabbit ovarian cells both *in vivo* and *in vitro*. This observation corresponds to a previous report on the ability of cAMP regulators to affect rat (HILLIER & TETSUKA 1997; AMSTERDAM *et al.* 2003; PELUSO 2006), human (AMSTERDAM *et al.* 2003) and porcine (SIROTKIN *et al*. 2010a; SIROTKIN 2005; MAKAREVICH *et al.* 2004a) ovarian steroidogenesis. Previously we failed to detect the influence of a cAMP/protein kinase A blocker, Rp-cAMP, on rabbit ovarian progesterone release *in vitro* (MAKAREVICH *et al.* 2000). On the other hand, our present observations are in line with a previous report on the influence of dbcAMP on rabbit ovarian secretory activity (CHRENEK *et al.* 2010). The physiological significance of dbcAMP-induced reduction in estradiol secretion *in vivo*, as well as of progesterone and estradiol release *in vitro* is not understood. Furthermore, direct or possible feedback mechanisms of cAMP-induced changes in steroid hormones remain unknown. Nevertheless, the importance of these hormones in the control of ovarian folliculo- and oogenesis, atresia, proliferation and apoptosis (HILLIER & TETSUKA 1997; HILLIER 2001; PELUSO 2006) and oviduct functions (ORIHUELA *et al.* 2003; DOUGHERTY & SANDERS 2005) suggest that changes in these processes induced by promoters of cAMP-dependent intracellular mechanisms reported previously could be mediated by changes in production of steroid hormones and/or their regulators. Similarity of the main dbcAMP effects *in vivo* and *in vitro* demonstrate that ovarian cells may be the main site of dbcAMP action, although some differences in *in vivo* and *in vitro* effects observed in our experiments also suggest the involvement of cyclic nucleotide in control of upstream regulators of ovarian functions.

The action of FSH, IGF-I and ghrelin on the release of steroid hormones and on the accumulation of proliferation and apoptosis markers in ovarian cells observed in our *in vitro* experiments confirm previous data in rabbits (CHRENEK *et al.* 2010; SIROTKIN *et al*. 2008b; SIROTKIN *et al*. 2009) and in other mammals (HILLIER 2001; SIROTKIN 2005; PELUSO 2006). Furthermore, the similar stimulatory action of dbcAMP, FSH and ghrelin on accumulation of PCNA, caspase 3 and bax, and similar inhibition of dbcAMP and hormones on progesterone release observed in our experiments may indicate that the action of these hormones on proliferation, apoptosis and progestogen release by rabbit ovarian cells could be mediated by cAMP-dependent intracellular mechanisms.

Moreover, in our experiments, administration of dbcAMP promoted the action of hormones on the accumulation of visible proliferation- and apoptosis-associated substances. This suggests that dbcAMP can increase the response of ovarian cells to hormonal stimulators. This increased response could be due to a cAMP-induced increase in hormone receptor expression reported previously (NAKAMURA 1995; TANO 1997). On the other hand, administration of dbcAMP did not promote the action of hormones on the release of steroids: it did not modify the effect of hormones on estradiol release and even inverted the action of hormones on progesterone and testosterone release from stimulatory to inhibitory. This observation suggests that cAMP does not mediate or support the action of peptide hormones on steroid hormones.

Taken together, our observations (1) confirm involvement of peptide hormones FSH, IGF-I and ghrelin in control of ovarian cell proliferation, apoptosis and steroid hormone release; (2) demonstrate the involvement of cAMP-dependent intracellular mechanisms in up-regulation of rabbit ovarian cell proliferation, apoptosis and in down-regulation of ovarian steroidogenesis; (3) suggest the involvement of cAMP-dependent mechanisms in mediating and/or promoting the effects of peptide hormones FSH, IGF-I and ghrelin on ovarian cell proliferation, apoptosis, but not on steroidogenesis; (4) suggest that the stimulatory action of dbcAMP on rabbit reproduction could be due to dbcAMP-induced changes in ovarian cell proliferation, apoptosis, steroid hormone release and in the response of these processes to hormonal regulators.

Acknowledgements

The authors thank MSc. Ž. KUKLOVÁ and Mrs. K. TOTHOVÁ for technical assistance.

References

AMSTERDAM A., SASSON R., KEREN-TAL I., AHARONI D., DANTES A., RIMON E., LAND A., COHEN T., DOR Y., HIRSH L. 2003. Alternative pathways of ovarian apoptosis: death for life. Biochem. Pharmacol. **66**: 1355-1362.

CHEADLE C., NESTEROVA M., WATKINS T., BARNES K.C., HALL J.C., ROSEN A., BECKER K.G., CHO-CHUNG Y.S. 2008. Regulatory subunits of PKA define an axis of cellular proliferation/differentiation in ovarian cancer cells. BMC Med. Genomics **1**: 43.

CHRENEK P., GROSSMANN R., SIROTKIN A. V. 2010. The cAMP analogue, dbcAMP affects release of steroid hormones by cultured rabbit ovarian cells and their response to FSH, IGF-I and ghrelin. Eur. J. Pharmacol. **640**: 202-205.

CHRENEK P., PIVKO J., MAKAREVICH A. V., KUBOVIČOVÁ H., BALAZI A., SIROTKIN A.V. 2012. The cAMP analogue, dbcAMP can stimulate rabbit reproductive functions. I. Effect on ovarian folliculogenesis, ovulation and embryo production. Acta Veterinaria (Beograd). **62**: 227-237.

DEGLI ESPOSTI M. 2004. Mitochondria in apoptosis: past, present and future. Biochem. Soc. Trans. **32**: 493-495.

DOUGHERTY D.C., SANDERS M.M. 2005. Estrogen action: revitalization of the chick oviduct model. Trends Endocrinol. Metab. **16**: 414-419.

FRANKS S., MASON H., WILLIS D. 2000 Follicular dynamics in the polycystic ovary syndrome. Mol. Cell. Endocrinol. **163**: 49-52.

GRAWES L.M., LAWRENCE J.C. 1996. Insulin, growth factors, and cAMP. Antagonism in the signal transduction pathways. Trends Endocrinol. Metab. **7**: 43-50.

HARADA H., GRANT S. 2003. Apoptosis regulators. Rev. Clin. Exp. Hematol. **7**: 117-138.

HILLIER S.G. 2001. Gonadotropic control of ovarian follicular growth and development. Mol. Cell. Endocrinol. **179**: 39-46.

HILLIER S.G., TETSUKA M. 1997. Role of androgens in follicle maturation and atresia. Baillieres Clin. Obstet. Gynaecol. **11**: 249-260.

HSUEH A.J., EISENHAUER K., CHUN S.Y., HSU S.Y., BILLIG H. 1996. Gonadal cell apoptosis. Recent Progr. Horm. Res. **51**: 433-455.

MAGA G., HUBSCHER U. 2003. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. J. Cell. Sci. **116**: 3051-3060.

MAKAREVICH A., SIROTKIN A. 2000. Presumptive mediators of growth hormone action on insulin-like growth factor I release by porcine ovarian granulosa cells. Biol. Signal. Recept. **9**: 248-254.

MAKAREVICH A., SIROTKIN A., CHRENEK P., BULLA J., HETENYI L. 2000. 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. J. Steroid. Biochem. Mol. Biol. **73:** 123-133.

MAKAREVICH A. V., SIROTKIN A. V., FRANEK J., KWON H.B., BULLA J. 2004b. The role of oxytocin, protein kinase A, and ERK-related MAP-kinase in the control of porcine ovarian follicle functions. Exp. Clin. Endocrinol. Diabetes. **112**: 108-114.

MAKAREVICH A.V., SIROTKIN A. V., GENIESER H.G. 2004a. Action of protein kinase A regulators on secretory activity of porcine granulosa cells in vitro. Anim. Reprod. Sci. **81**: 125-136.

MCKENNA S.D., PIETROPAOLO S.D., TOS E.G., CLARK A., FISCHER D., KAGAN D., BAO B., REDRESE P. J., PALMER S. 2005. Pharmacological inhibition of phosphodiesterase 4 triggers ovulation in follicle-stimulating hormone-primed rats. Endocrinology. **146**: 208-214.

MURPHY B.D., DOBIAS M. 1999. Homologous and heterologous ligands downregulate follicle-stimulating hormone receptor mRNA in porcine granulosa cells. Mol. Reprod. Dev. **53**: 198-207.

NAKAMURA M., NAKAMURA K., IGARASHI S., TANO M., MIYAMOTO K., IBUKI Y., MINEGISHI T. 1995. Interaction between activin A and cAMP in the induction of FSH receptor in cultured rat granulosa cells. J. Endocrinol. **147**: 103-110.

ORIHUELA P.A., PARADA-BUSTAMANTE A., CORTÉS P.P., GATICA C., CROXATTO H.B. 2003. Estrogen receptor, cyclic adenosine monophosphate, and protein kinase A are involved in the nongenomic pathway by which estradiol accelerates oviductal oocyte transport in cyclic rats. Biol. Reprod. **68**: 1225-1231.

PEEGEL H., TOWNS R., NAIR A., MENON K.M. 2005. A novel mechanism for the modulation of luteinizing hormone receptor mRNA expression in the rat ovary. Mol. Cell. Endocrinol. **233**: 65-72.

PELUSO J.J. 2006. Multiplicity of progesterone's actions and receptors in the mammalian ovary. Biol. Reprod. **75**: 2-8.

PELUSO J.J., PAPPALARDO A., WHITE B.A. 1993. Control of rat granulosa cell mitosis by phorbol ester-, cyclic AMP-, and estradiol-17beta-dependent pathways. Biol. Reprod. **49**: 416-422.

PIERRE A., PISSELET C., DUPONT J., MANDON-PÉPIN B., MONNIAUX D., MONGET P., FABRE S. 2004. Molecular basis of bone morphogenetic protein-4 inhibitory action on progesterone secretion by ovine granulosa cells. J. Mol. Endocrinol. **33**: 805-817.

POON S. L., AN B. S., SO W. K., HAMMOND G. L., LEUNG P. C. 2008. Temporal recruitment of transcription factors at the 3',5'-cyclic adenosine 5'-monophosphate-response element of the human GnRH-II promoter. Endocrinology **149**: 5162-5171.

RAMAKRISHNAPPA N., RAJAMAHENDRAN R., LIN Y. M., LEUNG P. C. 2005. GnRH in non-hypothalamic reproductive tissues. Anim. Reprod. Sci. **88**: 95-113.

SCHAEFFER H.J., SIROTKIN A. V. 1995. The release of insulin-like growth factor-I by luteinized human granulosa cells *in vitro*: Regulation by growth hormone, oxytocin, steroids and cAMP-dependent intracellular mechanisms. Exp. Clin. Endocrinol. Metab. **103**: 361-366.

SIROTKIN A.V., NITRAY J., KOLENA J., BULLA J. 1994. Cooperation between LH-RH and LH in the direct action on the ovary: LH-RH stimulation of LH/hCG receptors, basal and LH-induced cAMP and cGMP release by porcine granulosa cells in vitro. Cell. Signal. **6**: 135-140.

SIROTKIN A.V. 1996. Inter-relationships between nonapeptide hormones and cyclic nucleotides within cultured porcine granulosa cells. J. Endocrinol. **150**: 343-348.

SIROTKIN A. V. 2005. Control of reproductive processes by growth hormone: extra- and intracellular mechanisms. Vet. J. **170**: 307-317.

SIROTKIN A. V., CHRENEK P., ČURLEJ J., ZÁHRADNÍKOVÁ M. 2008a. Effect of 3-isobutyl-1-methyl-xanthine on rabbit superovulation and egg recovery. Slovak J. Anim. Sci. **41**: 57-59.

SIROTKIN A. V., CHRENEK P., DARLAK K., VALENZUELA F., KUKLOVÁ Z. 2008b. Some endocrine traits of transgenic rabbits. II. Changes in hormone secretion and response of isolated ovarian tissue to FSH and ghrelin. Physiol. Res. **57**: 745-751.

SIROTKIN A. V., RAFAY J., KOTWICA J., DARLAK K., VALENZUELA F. 2009. Role of ghrelin in regulating rabbit ovarian function and the response to LH and IGF-I. Domest. Anim. Endocrinol. **36**: 162-172.

SIROTKIN A. V., CHADIO S., CHRENEK P., XYLOURI E., FOTOPOULOY H. 2010a. Phosphodiesterase inhibitor 3-isobutyl-methyl-xanthine affects ovaria moprphology and stimulates reproduction in rabbits. Eur. J. Inflamm. **8**: 173-179.

SIROTKIN A.V., CHRENEK P., CHADIO S., XYLOURI E., FOTOPOULOY H., MAKAREVICH A.V. 2010b. Phosphodiesterase inhibitor 3-isobutyl-methyl-xanthine affects rabbit ovaries and oviduct. Eur. J. Pharmacol. **643**: 145-151.

SIROTKIN A.V, MAKAREVICH A.V., GROSMANN R. 2010c. Protein kinases and ovarian functions. J. Cell. Physiol. **226**: 37-45.

SPACZYNSKI R.Z., TILLY J.L., MANSOUR A., DULEBA A.J. 2005. Insulin and insulin-like growth factors inhibit and luteinizing hormone augments ovarian theca-interstitial cell apoptosis. Mol. Hum. Reprod. **11**: 319-324.

TANO M., MINEGISHI T., NAKAMURA K., KARINO S., IBUKI Y., MIYAMOTO K. 1997. Transcriptional and post-transcriptional regulation of FSH receptor in rat granulosa cells by cyclic AMP and activin. J. Endocrinol. **153**: 465-473.

VIEGAS L.R., HOIJMAN E., BEATO M., PECCI A. 2008. Mechanisms involved in tissue-specific apopotosis regulated by glucocorticoids. J. Steroid. Biochem. Mol. Biol. **109**: 273-278.