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Effect of cGMP analogues and protein kinase G blocker on secretory activity, apoptosis and the cAMP/protein kinase A system in porcine ovarian granulosa cells in vitro

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Abstract

The aim of the present study was to examine the role of cGMP-dependent intracellular mechanisms in control of ovarian functions. In the first series of experiments we studied the effects of the cGMP analogues 8-pCPT-cGMP (0.001–100 nM), Rp-8-pCPT-cGMPS (0.01–100 nM), Rp-8-Br-cGMPS (0.01–100 nM), and Rp-8-Br-PET-cGMPS (0.01–100 nM) on the release of progesterone, insulin-like growth factor I (IGF-I) and oxytocin by cultured porcine granulosa cells. In a second series of experiments, the effects of Rp-8-Br-PET-cGMPS (50 nM) and KT5822 (100 ng/ml), specific inhibitor of cGMP-dependent protein kinase (PKG), on cAMP, PKA, oxytocin and the occurrence of apoptosis in cultured cells were compared. The release of hormones and IGF-I into the culture medium was evaluated using a RIA, while the percentage of cells containing visible oxytocin, cAMP, as well as the regulatory and catalytic subunits of PKA was assessed using immunocytochemistry. Occurrence of apoptosis in these cells was detected using the TUNEL method. The stimulatory (8-pCPT-cGMP and Rp-8-pCPT-cGMPS), inhibitory (Rp-8-Br-cGMPS) and biphasic (Rp-8-Br-PET-cGMPS) effect of cGMP analogues on progesterone release was observed. All cGMP analogues used suppressed IGF-I release. All cGMP analogues decreased oxytocin release, but 8-pCPT-cGMP and Rp-8-Br-cGMPS, when given at low doses (0.01–0.1 and 1–10 nM, respectively) stimulated oxytocin output. Both, Rp-8-Br-PET-cGMPS and KT5822 increased the rate of incidence of apoptosis and percentage of cells containing immunoreactive cAMP. Both Rp-8-Br-PET-cGMPS and KT5822 decreased the proportion of cells containing immunoreactive oxytocin and regulatory subunit of PAK KT5822, but not Rp-8-Br-PET-cGMPS, increased the number of cells containing catalytic subunit of PKA. The present observations suggest the involvement of cGMP and PKG in control of the production of steroid, nonapeptide hormone, growth factor, cAMP and cAMP-dependent PKA, as well as the induction of apoptosis in porcine ovarian cells.

Keywords: Cyclic guanosine monophosphate; Insulin-like growth factor I; Oxytocin; Progesterone; Cyclic adenosine monophosphate; Protein kinase A; Protein kinase G

1. Introduction

There is evidence that guanosine-3′5′-cyclic monophosphate (cGMP) can be an important element of cellular signalling in various cell types. Extracellular stimuli via nitric oxide can influence formation of cGMP, which regulate functions of *myo*-, adipocytes and other cells in non-reproductive systems [1–3]. The role and mechanisms of action of cGMP in reproductive processes is, however, studied insufficiently. There is evidence that cGMP may regulate reproductive processes and mediate the action of some hormones and related substances on the reproductive system. At least, cGMP analogues or activators of endogenous cGMP production are able to activate progesterone and estra-

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diol secretion (rat [4], pig [5]), suppress spontane and LH-stimulated oocyte maturation (rat [6]) and apoptosis in ovarian cells (rat [7]). On the other hand, it has been reported [8], that cGMP formation in bovine ovarian cells can be stimulated by tumour necrosis factor α — a known inducer of apoptosis. Some authors failed to detect any effect of cGMP agonists on steroid hormones (rat [9], women [10]) release. Thus, available evidence on the effects of cGMP and related substances in the control of reproductive processes is still incomplete and there is no correspondence in the description of cGMP effects on ovarian apoptosis and steroidogenesis. The involvement of cGMP-dependent intracellular mechanisms in control of non-steroidal ovarian substances (nonapeptide hormones and growth factors) has not been studied yet.

With respect to the mechanisms of cGMP action, it is already known that it can regulate functions of non-ovarian cells through PKG, CGI and phosphodiesterases (PDE). cGMP is able to influence the cAMP/protein kinase A (PKA) system via PDE; cGMP may be a potent competitive inhibitor of cAMP-specific PDE and, therefore, can increase the level of intracellular cAMP [1–3].

The mechanisms of cGMP signalling within ovarian cells are still unknown. To understand, whether or not reproductive effects of cGMP are mediated by cGMP-dependent PKG, the action of cGMP analogues and regulators of PKG other than cGMP should be compared. No such comparative studies have been performed previously. In order to examine the involvement of the cAMP/PKA system in the mediation of cGMP/PKG effects, the action of cGMP analogues and other regulators regulators of PKG on cAMP and PKA should be investigated. Again, no such studies on ovarian cells have been reported until now.

In the first series of experiments we studied the influence of several cell membrane-permeable cGMP analogues on release of steroid (progesterone), nonapeptide hormone (oxytocin) and insulin-like growth factor I (IGF-I) into the culture medium. In the second series of experiments we attempted to understand the intracellular mechanisms of cGMP action on ovarian cells observed in the first series of experiments, (1) whether cGMP can affect the augmentation of hormone within the cells or rather hormone output from the cells; (2) whether cGMP can control not only hormones and growth factor, but also hormone- and growth factor-dependent apoptosis; and (3) whether cGMP can affect ovarian function through cAMP, cAMP-dependent PKA- and cGMP-dependent PKG. For this purpose we compared the effects of one cGMP analogue, Rp-8-Br-PET-cGMPS and direct PKG blocker, KT5822 on the presence of oxytocin, TdT (indicator of fragmented DNA and apoptosis), cAMP and two main subunits of cAMP-dependent PKA within porcine ovarian cells.

2. Materials and methods

2.1. Preparation, culture and processing of granulosa cells

The content of 2–5 mm follicles was aseptically aspirated from ovaries of Slovakian white gilts 180–240 days of age, killed at a local slaughterhouse at the early and mid-follicular phase of cycle. The suspension of granulosa cells was isolated and cultured as described previously [11].

In the first series of experiments (effect of cGMP analogues on release of substances into the medium) experimental groups received the following membrane permeable and PDE resistant cGMP analogues produced by BIOLOG Life Science Institute (Bremen, FRG), 8-pCPT-cGMP (0.001, 0.01, 0.1, 1, 10 or 100 nM), Rp-8-pCPT-cGMPS (0.01, 0.1, 1, 10, 100 nM), Rp-8-Br-cGMPS (0.01, 0.1, 1, 10 or 100 nM) or Rp-8-Br-PET-cGMPS (0.01, 0.1, 1, 10 or 100 nM).

The testing of these cGMP analogues on non-ovarian cells of rat, human [12,13], salamander and frog [14–16] demonstrated that 8-pCPT-cGMP may be an activator of cGMP-dependent protein kinases 1-α and 1-β and also of type II and cGMP-dependent ion channels. Rp-8-pCPT-cGMPS and Rp-8-Br-cGMPS may be as inhibitors of PKG and stimulators of CGI, while Rp-8-Br-PET-cGMPS can block both, PKG and CGI. These compounds do not affect PDE 2 or 3 to a significant extent, although Rp-8-Br-PET-cGMPS has moderate inhibitory properties for PDE 5. The action of these cGMP analogues on PKG, CGI and PDE in porcine ovarian cells is unknown. These cGMP analogues were dissolved immediately before the experiment in the incubation medium mentioned above. Control groups were represented with cells cultured without additions of treatments and with incubation medium cultured without cells (blank control). After 2 days of culture (time of high hormone and growth factor release) the cells and incubation medium from plate wells was gently aspirated and frozen at −18°C for RIA, gel electrophoresis and immunoblotting.

In the second series of experiments (effect of cGMP analogue and of PKG blockers on the presence of cAMP, PKA, oxytocin and apoptosis in cultured cells), experimental treatments consisted of Rp-8-Br-PET-cGMPS (50 nM) or KT5822 (Calbiochem-Novabiochem Corp., La Jolla, USA, 100 ng/ml), specific inhibitor of PKG, but not of CGI. Rp-8-Br-PET-cGMPS was dissolved immediately before the experiment in incubation medium, while KT5822 was first dissolved in 50 μl of DMSO to reach the concentration of 1 mg/ml. Immediately before an experiment, this stock solution of KT5822 was dissolved in incubation medium so that the content of DMSO did not exceed 0.001% of medium. Control cells were cultured without

Rp-8-Br-PET-cGMPS or KT5822. After 6 days in culture (time of formation of cell monolayer, which is suitable for immunocytochemical study, and time of good expression of apoptosis in granulosa cell culture according to our previous observations), chamber-slides were washed three times in ice-cold PBS, fixed for 3 h at −18°C with pure aceton (Lachema, Brno, Czech Republic), air-dryed and kept by +4°C to await immunocytochemical analysis.

In both series of experiments, the number of cells and viability after culture were determined by Trypan blue staining and a haemocytometer. No statistically significant differences between these indices in control and experimental groups were observed.

2.2. Immunoassay

Concentrations of substances in 25–100 ul of incubation medium were determined by RIA.

Progesterone was analysed using commercial RIA kits from DSL (Webster, TX, USA) according to the instruction of manufacturer. Sensitivity of analysis was 0.12 ng/ml. The cross-reactivity of the antiserum used, to pregnenolone, androstenediol, testosterone, estradiol and cortisol was less than 0.001%. Inter- and intraassay coefficients of variation did not exceed 1 and 8%, respectively.

Oxytocin was determined using our RIA as described previously [17]. Rabbit antiserum against oxytocin kindly provided by Dr G. Kotwica (University of Agriculture and Technology, Olsztyn, Poland) showed less than 0.01% cross-reactivity with arginine- and lysine-vasopressin, vasotocin, angiotensin and somatostatin. The sensitivity of the method was 3 pg/ml. Inter- and intrassay coefficients of variation were 14.6 and 7.5%, respectively.

IGF-I was measured according to the method described previously [18]. Anti-IGF-I antiserum from Peninsula Laboratories, (Belmont, USA) had a cross-reactivity of less than 0.01% to insulin, proinsulin, EGF, oxytocin, pGH and 1.9% to IGF-II. Sensitivity of assay was 1.4 pg/ml, inter- and intraassay coefficients of variation were less than 3.3 and 8.2%, respectively.

2.3. Immunocytochemical analysis

The granulosa cells fixed at chamber-slides were processed by immunocytochemistry [19]. For detection of PKA, primary mouse monoclonal antibodies against the regulatory subunit I (dilution 1:1000) and catalytic subunit C-α (dilution 1:250) of PKA (Transduction Laboratories, Lexington, USA) were used. 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 were confirmed prior to the experiments by Western blotting (see below). For detection of cAMP primary rabbit polyclonal anti-cAMP antiserum kindly provided by S. Greenhut, national hormone and pituitary program, NIH, Rockville, USA at the dilution 1:100 was used. This antiserum did not express species-specific differences in binding to bovine, porcine and rabbit ovarian cells and no binding to 5′-AMP, 5′-ADP, 5-ATP or 3′,5′-cGMP. For demonstration of oxytocin, sheep antiserum against oxytocin kindly provided by Professor A.P.F. Flint (University of Nottingham, Sutton Bonington, UK) at the dilution 1:100 was used. For visualisation of primary antibodies, a secondary polyclonal antibody against mouse, rabbit or sheep IgGs labelled with horseradish peroxidase (Sevac, Prague, Czech Republic or Transduction Laboratory; dilution 1:500) and DAB-reagent (Boehringer Mannheim GmbH, Mannheim, Germany; 10%) were used. As a negative control the cells treated with secondary antibodies and DAB, but not with primary antibodies were used. Presence of PKA, cAMP or oxytocin immunoreactivity in each cell was examined by light microscopy.

2.4. Protein gel electrophoresis and immunoblotting

Frozen lysates of granulosa cells were processed and subjected to SDS-polyacrylamide gel electrophoresis according to Laemmli [20] The PKA was evaluated using immunoblotting [21] and mouse monoclonal antibodies against the catalytic and regulatory subunits of PKA described above (1:250 and 1:1000, respectively).

2.5. TUNEL analysis

Detection of apoptotic cells (containing fragmented DNA) after culture in chamber-slides was performed by TUNEL (TdT-mediated dUTP nick end labelling) method using an in situ cell death detection kit and DAB reagent from Boehringer Mannheim, GmbH according to the instruction of the manufacturer. As a negative control fixed and permeabilized cells incubated without TdT, but with secondary antibodies and DAB were used. Cells incubated 10 min at room temperature with bovine pancreatic DNAse I (Boehringer Mannheim GmbH, 0.01 mg/ml) before TdT treatments were used as a positive control. Presence of TUNEL in each cell was determined by light microscopical inspection of cultures.

2.6. Statistics

Each experimental group was represented by four culture wells or chambers. RIA of hormone and growth factor level were performed in duplicate in incubation mediums from each plate well, while proportion of cells containing specific immunoreactivity (immunocytochemical and TUNEL analysis) was counted on the

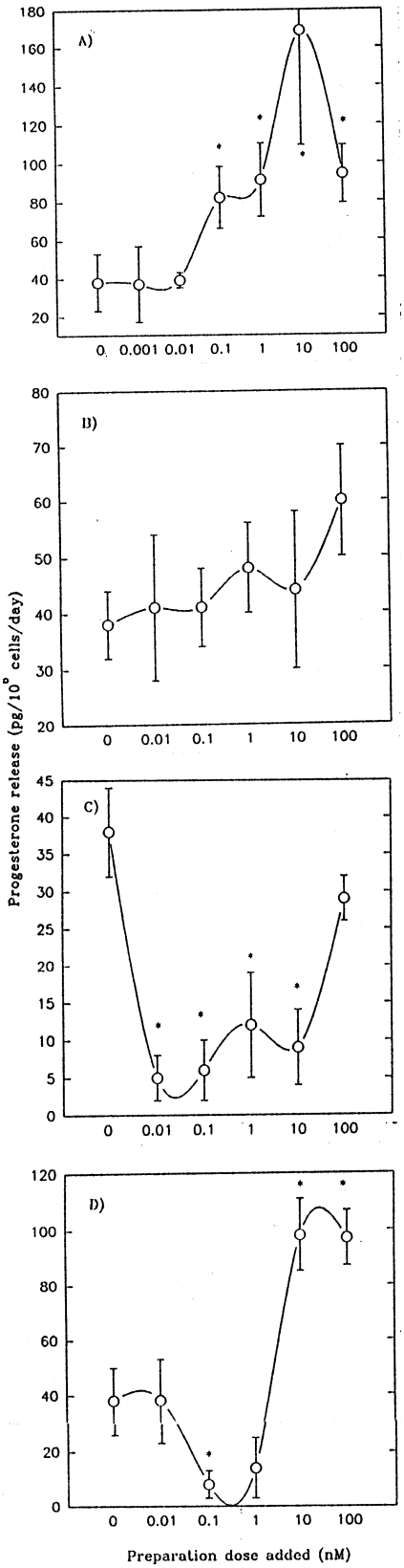

Fig. 1. (a) Effect of 8-pCPT-cGMP, (b) Rp-8-pCPT-cGMPS, (c) Rp-8-Br-cGMPS and (d) Rp-8-Br-PET-cGMPS on progesterone release by cultured porcine granulosa cells. Values are mean ± S.E.M. ($n = 12$ culture wells); *, significant ($P < 0.05$) difference compared with control (medium without addition).

basis of inspection of 1000 cells minimum per chamber. The data shown are means of values obtained in three separate experiments performed, using separate pools of granulosa cells obtained from 20 to 40 animals. The quantities of hormones and IGF-I released by cells were calculated as the differences between the values determined in the medium incubated with and without (blank control) granulosa cells. The rates of release were calculated per 10^6 viable cells per day. Significant differences between the experiments were evaluated using two-way ANOVA. When ANOVA indicated effects of treatments, RIA data from experimental and control groups were compared with Duncan's test. By immunocytochemical and TUNEL analysis, significant differences in the proportion of cells contained PKA, cAMP, oxytocin or TdT immunoreactivity were evaluated by the χ^2-test.

3. Results

RIA results of the incubation medium showed, that cultured porcine granulosa cells released significant amounts of progesterone, IGF-I and oxytocin (Figs. 1–5).

3.1. Effect of cGMP analogues on progesterone release by cultured granulosa cells

8-pCPT-cGMP significantly ($P < 0.05$) increased progesterone release when given at doses 0.1–100 nM with maximal effects at 10 nM. Lower doses of this substance (0.001 or 0.01 nM) did not affect progesterone accumulation in the medium (Fig. 1a).

Rp-8-pCPT-cGMPS expressed progesterone-stimulating properties when given at high dose (100 nM); the lower doses of this substance were without any effects (Fig. 1b).

In contrast to the results with 8-pCPT-cGMP and Rp-8-pCPT-cGMPS, Rp-8-Br-cGMPS inhibited progesterone output at doses 0.01–10 nM with maximal effects at 0.01 nM. The highest dose (100 nM) was without effect (Fig. 1c).

Rp-8-Br-PET-cGMPS, had a biphasic effect on progesterone release, the lowest dose (0.01 nM) gave no effect, medium dose (0.1 nM) significantly ($P < 0.05$) inhibited progesterone release, while higher doses (10 or 100 nM) dramatically stimulated it ($P < 0.05$) (Fig. 1d).

3.2. Effect of cGMP analogues on IGF-I release by cultured granulosa cells

8-pCPT-cGMP, when given at low doses (0.001–0.1 nM) did not substantially affect IGF-I release, while higher concentrations (1–100 nM) were inhibitory with maximal inhibitory effect at 1 nM (Fig. 2a).

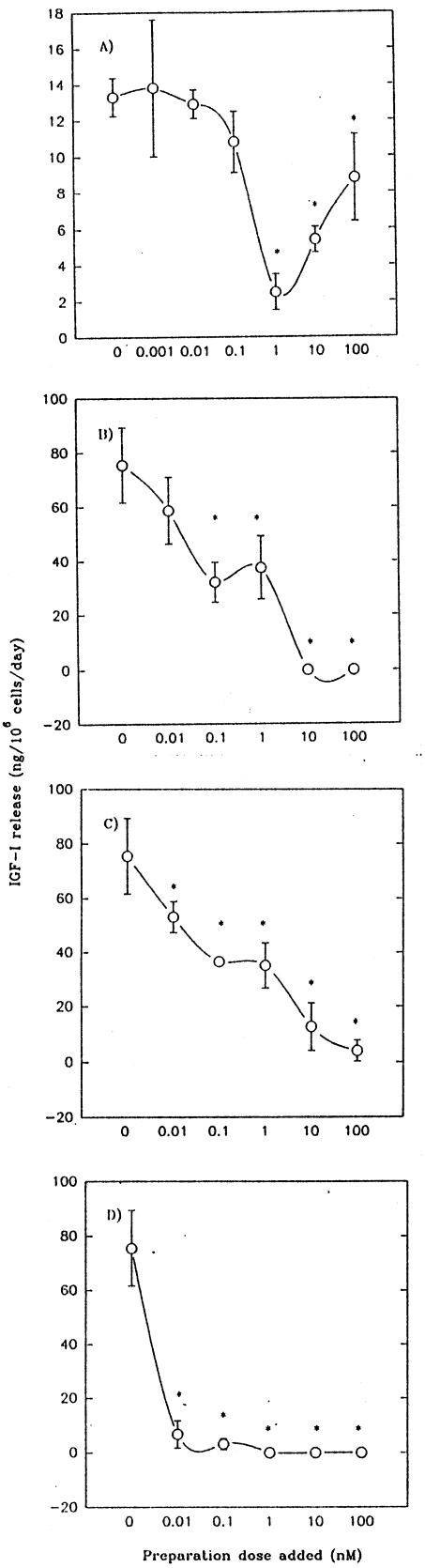

Fig. 2. (a) Effect of 8-pCPT-cGMP, (b) Rp-8-Br-cGMPS, (c) Rp-8-pCPT-cGMPS and (d) Rp-8-Br-PET-cGMPS on IGF-I release by cultured porcine granulosa cells. Values are mean ± S.E.M. ($n = 12$ culture wells); *, significant ($P < 0.05$) difference compared with control (medium without addition).

Rp-8-Br-cGMPS, RP-8-pCPT-cGMPS or Rp-8-Br-PET-cGMPS inhibited ($P < 0.05$) IGF-I accumulation at all doses used, but proportional to the dose added, with maximal effect at higher doses (1–100 nM). Moreover, Rp-8-Br-cGMPS and Rp-8-Br-PET-cGMPS were able to block IGF-I output completely (Fig. 2b–d).

3.3. Effect of cGMP analogues on oxytocin release by cultured granulosa cells

8-pCPT-cGMP had biphasic effect on oxytocin accumulation, when given at the lowest dose (0.001 nM) it did not affect oxytocin output at all, at higher doses (0.01 or 0.1 nM) it significantly ($P < 0.05$) increased, while at highest doses (1–100 nM) it significantly ($P < 0.05$) decreased oxytocin output with maximal suppressive effect at 100 nM (Fig. 3a).

Rp-8-Br-cGMPS also had a biphasic effect on oxytocin release, at doses 0.01 or 0.1 nM it did not affect hormone secretion, at doses 1 or 10 nM it dramatically ($P < 0.05$) stimulated it, while a dose of 100 nM was inhibitory ($P < 0.05$) (Fig. 3b).

Both, Rp-8-pCPT-cGMPS and Rp-8-Br-PET-cGMPS significantly ($P < 0.05$) decreased oxytocin release at doses 1–10 or 1 nM, respectively. Higher or lower doses of these preparations did not substantially influence oxytocin accumulation (Fig. 3c and d).

3.4. Effect of cGMP analogue and inhibitor of PKG on the presence of oxytocin, cAMP, the PKA subunits and on apoptosis in cultured granulosa cells

Immunocytochemical analysis of granulosa cells demonstrated, that porcine granulosa cells contain oxytocin, which release was confirmed by previous oxytocin RIA in culture medium. Furthermore, it demonstrated the presence of immunoreactivities corresponding to catalytic and regulatory subunits of PKA. Furthermore, a significant proportion of cultured bovine granulosa cells contained immunoreactivity corresponding to cAMP. TUNEL analysis demonstrated, that significant proportion of these cells at the end of culture have visible signs of apoptosis and DNA fragmentation — binding of TdT in the nuclei (Figs. 4 and 5, Table 1).

Both Rp-8-Br-PET-cGMPS (50 nM), and KT5822 (100 ng/ml), dramatically ($P < 0.05$) decreased the proportion of cells which bind antiserum against oxytocin, i.e. containing visible amounts of this nonapeptide hormone.

Furthermore, both Rp-8-Br-PET-cGMPS and KT5822, when given at these doses, significantly ($P < 0.05$) increased the proportion of cells containing immunoreactive cAMP.

Rp-8-Br-PET-cGMPS significantly ($P < 0.05$) decreased the proportion of cells containing immunoreac-

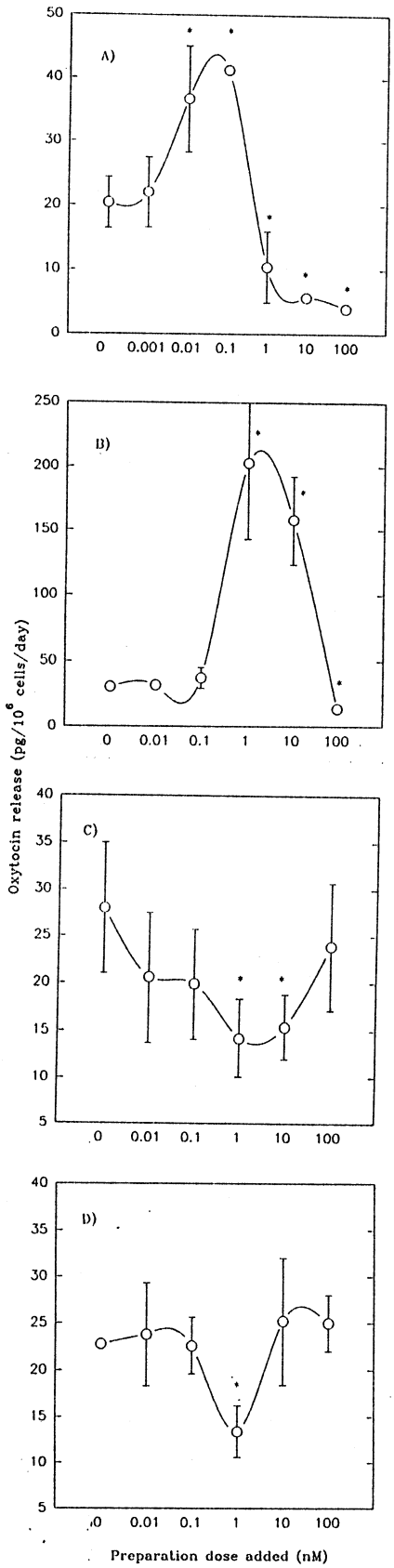

Fig. 3. (a) Effect of 8-pCPT-cGMP, (b) Rp-8-Br-cGMPS, (c) Rp-8-pCPT-cGMPS and (d) Rp-8-Br-PET-cGMPS on oxytocin release by cultured porcine granulosa cells. Values are mean ± S.E.M. ($n = 12$ culture wells); *, significant ($P < 0.05$) difference compared with control (medium without addition).

tive regulatory subunit of PKA, while this cGMP analogue only insignificantly increased the percentage of cells revealing PKA catalytic subunit.

KT5822 dramatically ($P < 0.05$) decreased the proportion of cells containing immunoreactive regulatory PKA subunit, but it significantly ($P < 0.05$) increased the relative number of cells revealing catalytic subunit of PKA.

Both Rp-8-Br-PET-cGMPS and KT5822 significantly ($P < 0.05$) increased also the rate of incidence of apoptosis in cultured porcine granulosa cells (Table 1).

4. Discussion

The present observations confirm results of previous reports [5,22–27] with respect to the production of progesterone, IGF-I, oxytocin and cAMP by cultured porcine granulosa cells. In case of oxytocin, the RIA data (first series of experiments) were confirmed by immunocytochemical data (second series of experiments), whose were seems not previously obtained on porcine granulosa cells. The present observation is probably the first demonstration of apoptosis and of PKA subunits in this system. Some differences in basal IGF-I and oxytocin output are observed in different experiments may be due to variability in material used (animals, stage of cell luteinization a.o.).

Our observations are in concert with previous data on the ability of cGMP analogues to stimulate progesterone secretion in rat [4] and porcine [5] ovarian cells and to promote apoptosis in rat ovarian cells [7]. This is probably also the first report on the involvement of cGMP and related messengers in the control of ovarian nonapeptide hormone, growth factor and the cAMP/PKA system. Our observation that Rp-8-Br-PET-cGMPS decreases oxytocin accumulation in both, the cells (oxytocin synthesis) and in the incubation medium (oxytocin release) suggests that cGMP analogues affect hormone release via its intracellular synthesis or metabolism.

Therefore, our data demonstrate an important role for cGMP and related substances in the control of the production of steroid, nonapeptide hormone, growth factor, cAMP, cAMP-dependent PKA and the survival of mammalian ovarian cells. It is well documented [6,7,17,25,27–29], that progesterone, oxytocin, IGF-I, cAMP and related PKA are the key regulators and/or indicators of ovarian follicular growth, ovulation, luteinization, atresia and of oocyte maturation. Therefore, cGMP-dependent intracellular mechanisms in the control of reproduction seem to be much more important, than it was supposed before. Since LH–RH, gonadotropins [7,24,30], GH, prolactin [31], oxytocin [25,32,33], steroid hormones [22,32] and melatonin [23] are potent activators of cGMP production and related

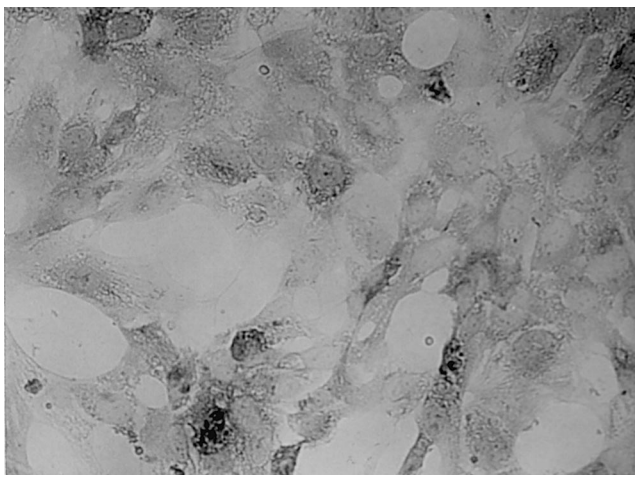

Fig. 4. Oxytocin identified using immunocytochemistry in the cytoplasm (dark staining) of cultured porcine granulosa cells (× 600).

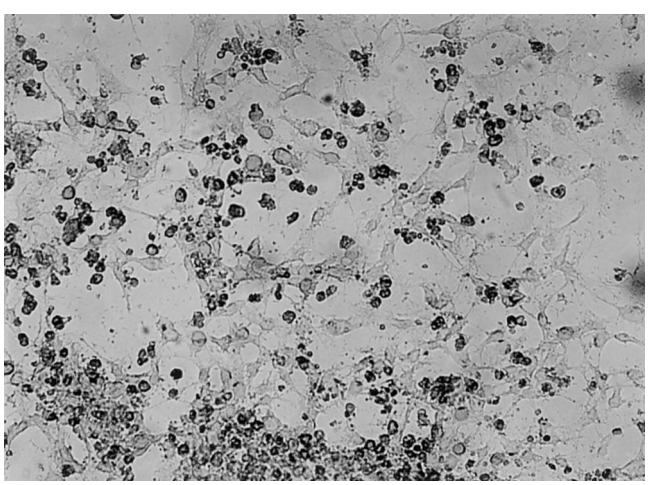

Fig. 5. TdT (indicator of apoptosis) identified using TUNEL method in the nuclei (dark staining) of cultured porcine granulosa cells (× 200).

intracellular messengers, the cGMP-dependent cascade may mediate hormonal signals within the ovary.

Some of the effects observed with the cGMP analogues and KT5822 on ovarian cells may be primary and direct, while other events may be secondary ones, and may reflect feedback relationships between the ovarian effectors. For example, IGF-I and oxytocin can be reciprocal stimulators, as well as activators of progesterone and cAMP production within the ovary [25–27]. Ovarian apoptosis can be regulated by IGF-I, steroid hormones and cAMP [7,21,28,29]. Steroid hormones can also stimulate or inhibit the production of cAMP [22,32], which, in turn, stimulate nonapeptide hormone [25] and IGF-I [34] release by ovarian cells. Interrelationships between ovarian substances therefore require further studies.

In non-ovarian cells three basic mediators of cGMP action are known, (1) PKG; (2) CGI; and (3) PDE which can affect cAMP or cGMP [1–3]. A comparison to the effects of various substances with different mechanisms of action in our experiments might potentially provide some hypotheses on possible mechanisms of cGMP action within the ovary. For example, no substantial association between pattern of influence of cGMP analogues on PKG, and CGI (determined previously) and release of progesterone, IGF-I and oxytocin (detected in the first series of our experiments) was found, cGMP analogues, whose were able both stimulate and inhibite PKG and CGI, stimulated progesterone and oxytocin and inhibited IGF-I output. This fact, together with the lack of correlation between the effects of Rp-8-Br-PET-cGMPS, which blocks both PKG and CGI in no-ovarian cells, and of KT5822, which blocks only PKG, observed in the second series of experiments, suggests that cGMP action is probably not mediated by PKG or CGI. On the other hand, this speculation is based on the information on the effect of cGMP analogues on PKG and CGI in non-ovarian and non-porcine cells, whilst effects of these cyclic nucleotides on PKG and CGI are unknown. Therefore, our data demonstrate the involvement of cGMP and PKG in control of ovarian cells, but not functional interrelationships between cGMP, PKG and CGI in these cells.

The influence of both Rp-8-Br-PET-cGMPS and of KT5822 on cAMP and cAMP-dependent PKA within the cells, observed in our experiments, demonstrates the involvement of the cAMP/PKA system in the action of cGMP and PKG or, at least, the expressed cross-talk between these two systems. In non-ovarian cells, cGMP is able to augment cAMP within the cells through competitive binding of cAMP-specific PDE [2,3]. Neither Rp-8-Br-PET-cGMPS nor KT5822 used in our

Table 1
Effect of cGMP analogue, Rp-8-Br-PET-cGMPS and of KT5822, inhibitor of PKG, on content of immunoreactive oxytocin, cAMP, the regulatory and catalytic subunits of PKA and on occurrence of apoptosis in cultured porcine granulosa cells[a]

Treatment	Proportion (%) of cells containing visible				
	Oxytocin	CAMP	PKA (regulatory subunit)	PKA (catalytic subunit)	TdT (apoptosis)
Control	75+3.4	21+5.7	80+3.7	45+4.7	31+2.6
Rp-8-Br-PET-cGMPS	0*	32+3.8*	67+3.3*	49+4.8	70+5.6*
KT5822	16+3.5*	32+2.9*	47+6.6*	64+2.2*	49+6.7*

[a] Values are mean ± S.E.M. ($n \leq 1000$ cells); *, significant ($P < 0.05$) differences with control (without addition).

experiments are able to affect PDEs [12–16]. Therefore, the effect of these compounds on cAMP is probably not due to the influence on PDE-induced cAMP catabolism. Furthermore, the phenomenon of cAMP accumulation under the influence of both Rp-8-Br-PET-cGMPS and KT5822, a structure, which is not related to cGMP, suggests the existence of an alternative mechanism of cGMP action on cAMP, which may include a cGMP/PKG rather than a cGMP/PDE axis. The augmentation of catalytic (biological active) and reduction in regulatory (biological inactive and degraded after activation of PKA; [35]) subunit of PKA observed, is probably the consequence of Rp-8-Br-PET-cGMPS- and of KT5822-induced accumulation of cAMP within the cells. Therefore, cGMP may influence ovarian functions through induction of the following possible sequence of events, cGMP → PKG? → cAMP → PKA → protein phosphorylation → cell response.

Acknowledgements

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