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ORIGINAL ARTICLE

# Different signaling in pig anterior pituitary cells by GNRH and its complexes with copper and nickel

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**Abstract**

Gonadotropin releasing hormone (GnRH) is an essential factor in the regulation of synthesis and release of pituitary gonadotropins. After binding to specific receptors and coupling with G proteins, it triggers the intracellular signaling involving the synthesis of inositol phosphates and diacylglycerol. Previously we have showed that certain metal complexes with GnRH, i.e. copper (Cu-GnRH) and nickel (Ni-GnRH) are able to bind to the GnRH receptors. The intracellular signalling of these complexes, however, has not been yet elucidated. In this experiment, the ability of the Cu-GnRH and Ni-GnRH complexes to modulate cAMP synthesis and phosphoinositols formation in the pig anterior pituitary cells *in vitro* was studied. The native GnRH and its metal complexes stimulated the luteinizing hormone (LH) release. but, only the effect of Cu-GnRH was found to be a dose-dependent. The metal complexes did not significantly influence inositol phosphates accumulation, while their effect on cAMP synthesis was significantly more potent than that of GnRH alone. We conclude that the Cu-GnRH and Ni-GnRH complexes increase LH release in the porcine pituitary cells although their intracellular signaling is different from that of the native GnRH. It seems that metal complexes with GnRH deserve more attention in further studies.

## Introduction

Hypothalamic neurohormone GnRH is a key substance in the regulation of biosynthesis and release of pituitary tropic hormones: luteinizing hormone (LH) and follicle stimulating hormone (FSH) which, in turn, influence ovarian gametogenesis and steroidogenesis. GnRH is periodically released to the portal vessels and reaches specific GnRH receptors on gonadotrope cells. This induces the intracellular events leading to the biosynthesis and release of LH and FSH. GnRH receptors possess seven transmembrane domains that are associated with G proteins and intracellular sig-

naling involving the inositol phosphates (IPs) synthesis. Formation of IPs initiates the cascade of events leading to the biosynthesis and release of LH and FSH [11, 18, 21].

It is known that metals may influence reproductive processes. Barnea and coworkers [1–5] showed that a complex of copper with certain amino acids and short peptides stimulates the release of GnRH from the hypothalamic secretory granules, both from isolated granules and tissue explants. It was also found that copper exhibits a stimulatory effect on the pituitary level [13].

The effect of copper, nickel and zinc complexes with GnRH on the release of LH and FSH *in vivo* was studied in our previous experiment [16]. As we have demonstrated, metal complexes with GnRH were more potent stimulators of gonadotropin release than the native GnRH. The receptor binding study of copper, nickel, zinc and cobalt complexes with GnRH revealed that only the copper complex had higher affinity to the rat and sheep pituitary receptors, while the remaining complexes showed lower affinity in comparison to the native GnRH [9, 15]. Therefore, we became interested in evaluating whether the signalling pattern of native GnRH and its copper as well as nickel complexes are similar in porcine pituitary cells.

In the current study, in order to ascertain the possible differences in the intracellular signalling, we examined the effect of GnRH and its copper and nickel complexes on the release of LH, inositol phosphates formation and intracellular synthesis as well as extracellular accumulation of cAMP in porcine pituitary cells.

## Materials and Methods

### *Chemicals*

GnRH was purchased from Sigma Aldrich (St. Louis, MO, USA). All other reagents were of the highest grade commercially available. Dulbecco's Modified Eagle's Medium (DMEM), McCoy's 5A Medium, BSA fraction V, theophylline, nystatin, gentamycin, bacitracin, 3-isobutyl-1-methylxanthine (IBMX), forskolin, biotinamidocapronate N-hydroxysuccinimide ester, streptavidin-peroxidase, 3,3',5,5'-tetramethylbenzidine, dimethylsulfoxide, 2'-O-monosuccinyladenosine-3',5'-cAMP monophosphate tyrosyl methyl ester for iodination and cAMP standard were purchased from Sigma (St. Louis, MO, USA). $^{125}$INa was obtained from POLATOM (Swierk, Poland). Myo-[$^{3}$H]inositol was purchased from NEN$^{TM}$ (Boston, MA, USA). Sephadex G-25 was obtained from Pharmacia (Uppsala, Sweden), and Dowex resin was from Bio-Rad (Hercules, CA, USA). Trypsin (0,25 % solution), fetal calf serum (FCS) and horse serum were obtained from BIOMED Vaccine Laboratory (Lublin, Poland).

### *Isolation and culture of porcine pituitary cells*

Pituitary glands were obtained from ten crossbred (Large White x Polish Landrace) mature gilts during the pre-ovulatory phase of the estrous cycle. Pituitary cells were dispersed aseptically as described previously [25, 26]. Briefly, the anterior lobes were dissected from each pituitary, minced into small pieces (1–2 mm) and washed several times with DMEM. Single cell suspension of anterior pituitaries was then prepared by sequential 0.25% trypsin digestions at 37 °C for 8–10 min. The pituitary cells were repeatedly centrifuged at 800 × g for 8 min, washed with DMEM, suspended and counted in a hemocytometer. Cell viability (97–98%) was determined by trypan blue dye exclusion. Finally, pituitary cells were resuspended in McCoy's 5A medium containing 10% horse serum, 2.5% fetal calf serum (Gibco BRL), 240 IU/ml nystatin and 20 μg/ml gentamycin at a density of $5 \times 10^5$ cells/ml. One ml of dispersed cells was transferred to each culture dish of 24-well plates and pre-incubated for 72 hours at 37 °C in a humidified atmosphere (95% air:5% $CO_2$) to allow completion of attachment. The cells were then washed twice with fresh McCoy's 5A medium without serum. After the final wash, pituitary cells were incubated in 1 ml of bacitracin containing ($2 \times 10^{-5}$ M) serum-free McCoy's medium.

### *LH determination*

LH concentration in incubation media was established by EIA [20]. Briefly, porcine LH (USDA-pLH-I-1) was labeled with biotinamidocapronate N-hydroxysuccinimide ester and dialysed overnight in the presence of 0.1% BSA in PBS. Standard curve (USDA-pLH-B-1) ranged from 3.12 to 50 ng/ml. All samples and standards were incubated for 18 h at 4°C with 100 μl of SZ/Z/89/370 [28] antiserum (1:200 000). Samples were then incubated at 4°C for 2 hours with biotinyl-porcine LH (2 ng/0.1 ml EIA buffer) and then the same concentration of biotinyl-porcine LH was added for the next 15 min. Plates were washed with ice-cold 0.01% Tween-80, and incubated in the dark at 25°C for 50 min with 150 μl substrate solution per well (substrate solution: 25 ml of 0.1 M $CH_3COONa$ with citric acid ; pH 5.5, plus 100 μl 1% $H_2O_2$ plus 400 μl 0.6% 3,3',5,5'-tetramethylbenzidine in dimethylsulfoxide). The reaction was stopped with 2 M $H_2SO_4$ and the intensity of coloration measured at 450 nm with a multichanel microtitration plate photometer (Labsystems, Helsinki, Finland). The intra- and interassay coefficient of variation was 3.75 and 4.24%, respectively.

To study dose-depedent effect of GnRH, Cu-GnRH and Ni-GnRH on LH secretion pituitary cells were incubated for 180 min with following doses of agents:$10^{-10}$, $10^{-9}$, $10^{-8}$ and $10^{-7}$M.

### *cAMP determination*

The influence of GnRH or the Cu-GnRH and Ni-GnRH complexes on cAMP production was determined in pituitary cells pre-incubated for 30 min with 100 μM phosphodiesterase inhibitor IBMX. Parallel incubations with 10 μM forskolin (an adenylate cyclase activator) served as a positive control. All incubations were performed in duplicates. Incubation media were collected, mixed 1:1 (v/v) with theophylline, boiled for 5 min and stored at –40 °C to complete an extracellular cAMP assay. Cells were washed with PBS, scraped in 80% ethanol for 1 min and centrifuged at 500 × g for 15

min. Supernatants were dried, resuspended with 0.25 ml of PBS and stored at –70 °C for the assay of intracellular cAMP.

The intra- and extracellular cAMP was radioimmunoassayed as described by Brooker et al. [7]. The 2'-O-monosuccinyl-cAMP was labeled with $^{125}$INa (1 mCi). The standard curve ranged from 0.45 to 500 fmol cAMP/0.1 ml PBS (pH 7.3) containing 0.1% BSA. Before the assay, samples and standards were acetylated with triethylamine/acetic anhydride (2:1, v/v) at room temperature in a fume hood. All acetylated samples were then incubated for 18 h at 4°C with $^{125}$INa and anti-cAMP (1:70 000). Then 0.5% (w/v) γ-globulin and 16% (w/v) polyethylene glycol was added, the samples were centrifuged at 1000 × g for 30 min and the radioactivity of pellets was measured. The intra- and interassay coefficients of variation were 2.08 and 2.19%, respectively.

***Phosphoinositide hydrolysis***

Phosphoinositide hydrolysis was determined according to the method described previously [21–23, 27]. Briefly, pituitary cells were incubated for three days in McCoy's medium containing myo-[$^3$H]inositol (1 μCi/ml medium). Next, cells were washed 3 times with serum-free McCoy's medium and incubated for 30 min with 10 mM LiCl and then treated for 30 min with GnRH or Cu-GnRH and Ni-GnRH. At the end of incubation, the medium was removed and 0.5 ml of ice-cold mixture of methanol and HCl (10:0.1 v/v) was added to each vial. Cells were harvested by scraping, and transferred to glass tubes containing 0.25 ml chloroform. After 30 min on ice, 0.4 ml chloroform and 0.4 ml 0.5 M EDTA were added and all samples were centrifuged at 1000 x g for 5 min. The upper aqueous phase was collected and stored at –20°C until chromatography analysis.

Inositol phosphates (IPs) were resolved by using anion exchange chromatography on Dowex resin columns (AG 1-X8, formate form; [21, 27]). Before use, columns were washed with 10 ml of 5 mM sodium tetraborate and then the samples were applied and 15 ml of distilled water was used to elute unincorporated inositol. Next, columns were sequentially washed with 15 ml of 5 mM sodium tetraborate-60 mM ammonium formate, 0.1 M formic acid-0.2 M ammonium formate, 0.1 M formic acid-0.4 M ammonium formate and 0.1 M formic acid-1 M ammonium formate. Effluents were collected in 5 ml fractions. The four eluates contained glycerophosphoinositol, inositol monophosphates ($IP_1$), inositol bisphosphates ($IP_2$) and inositol trisphosphates ($IP_3$), respectively. Two ml aliquots were mixed with 10 ml of scintillation liquid and counted on a β-counter. Data are presented as total IPs ($IP_1 + IP_2 + IP_3$).

***Statistical analysis***

All data are expressed as mean ± SEM. Differences between means were assessed by ANOVA, followed by Bonferonni test (GraphPad PRISM; GraphPad Software, Inc., San Diego, CA).

## Results

***Effect of GnRH and the Cu-GnRH and Ni-GnRH complexes on LH Secretion***

Time- and dose-dependent effects of GnRH, Cu-GnRH and Ni-GnRH on LH release are shown in fig. 1 and 2, respectively. Neither GnRH alone nor the metal-GnRH complexes affected the LH release during the 15 or 30 minute of incubation. In contrast, all treatments increased LH secretion during the 60 minute of pituitary cell incubation. At this time point, GnRH and Cu-GnRH exhibited a similar effect on LH secretion (158.5 and 157% of control value, respectively; P<0.05) while the Ni-GnRH was more effective (199% of control value; P<0.01) in stimulating LH secretion. A longer period of incubation (180 min) caused very high medium accumulation of LH in the presence of Cu-GnRH (230.7% of control value; P<0.01). The stimulating effect of GnRH and Ni-GnRH complexes on LH secretion was also significant, although slightly less pronounced than that of the Cu-GnRH (163 and 167% of the control value, respectively; P<0.01).

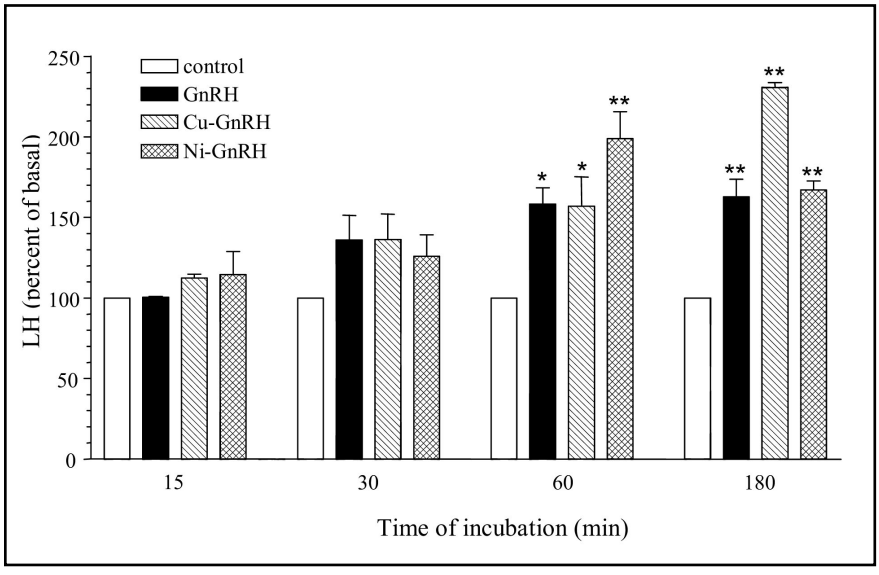


**Figure 1:** The effect of GnRH, Cu-GnRH and Ni-GnRH on LH release from porcine anterior pituitary cells during different times of incubation. Treatment concentrations were $10^{-7}$ M. *P<0.05; **P<0.01.

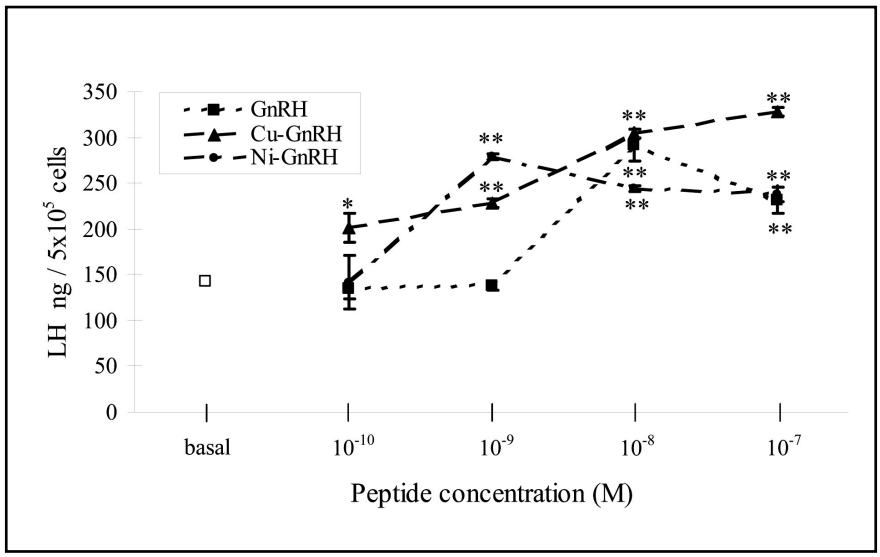


**Figure 2:** The effect of different doses of GnRH, Cu-GnRH and Ni-GnRH on LH release from porcine anterior pituitary cells. Incubation was performed for 180 minutes. *P<0.05; **P<0.01.

To study the dose-dependent effect of GnRH, Cu-GnRH and Ni-GnRH on LH secretion pituitary cells were incubated for 180 min. with following doses of treatments: $10^{-10}$, $10^{-9}$, $10^{-8}$ and $10^{-7}$ M (Fig. 2). At the lowest concentration, only Cu-GnRH increased LH secretion (201.62 ± 16 vs. 142.25 ± 9.04 ng LH/5 × $10^5$ cells; P<0.05). A higher concentration of the metal-GnRH complexes ($10^{-9}$ M) resulted in elevation of medium LH level (228.4 ± 4.6 and 279.3 ± 3.38 vs. 142.25 ± 9.04 ng LH/5 × $10^5$ cells, respectively; P<0.01). The native hormone had no effect. Both GnRH and the metal complexes stimulated LH release from pituitary cells at $10^{-8}$ M (P<0.01). Increasing the Cu-GnRH concentration to $10^{-7}$ M caused further elevation of LH release (328.21 ± 4.38 vs. 142.25 ± 9.04 ng LH/5 x $10^5$ cells; P<0.01). The highest concentration of GnRH and Ni-GnRH also stimulated LH secretion in comparison to basal LH output (231.8 ± 15.57 and 237.7 ± 8.17 vs. 142.25 ± 9.04 ng LH/5 × $10^5$ cells, respectively; P<0.01). All treatments exhibited their maximal LH releasing potency at different concentrations, i.e., GnRH at $10^{-8}$ M, Cu-GnRH at $10^{-7}$ M and Ni-GnRH at $10^{-9}$ M.

***Effect of GnRH, Cu-GnRH, and Ni-GnRH on cAMP Production***

Time-dependent effect of GnRH, Cu-GnRH, and Ni-GnRH on extracellular cAMP accumulation is presented in Figure 3. Neither GnRH nor the metal-GnRH complexes influenced cAMP release after 15 or 30 min of treatment. The effect of GnRH, Cu-GnRH, and Ni-GnRH on cAMP production was more pronounced after 60 min of incubation; however no statistical differences were observed when hormone-stimulated cAMP levels were compared to basal secretion of cAMP. Longer period of incubation (180 min) resulted in increased cAMP output from pituitary cells after treatment with native GnRH (184% of the control value; P<0.01) or the GnRH complexes with Cu (270% of the control value; P<0.01) and Ni (238% of the control value; P<0.01). Forskolin (10 μM; a positive control) added 10 min before the end of incubation stimulated cAMP secretion (P<0.001).

Intracellular cAMP concentration after one hour of pituitary cells incubation with $10^{-7}$ M of GnRH, Cu-GnRH and Ni-GnRH is depicted in Figure 4. The Cu-GnRH complex had the highest potency to induce

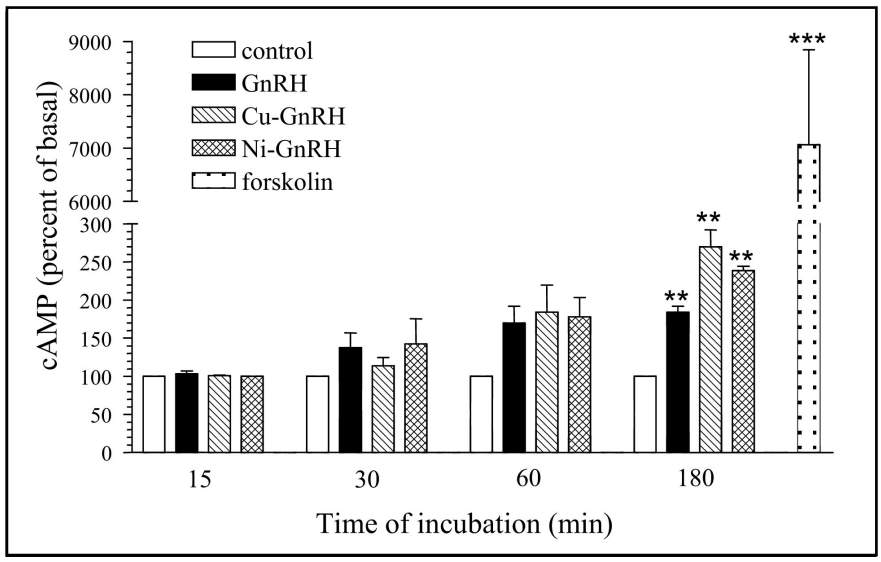


**Figure 3:** The effect of GnRH, Cu-GnRH and Ni-GnRH on extracellular cAMP in the porcine anterior pituitary during different times of incubation. Treatment concentrations were $10^{-7}$ M. Forskolin (10 μM; a positive control) was added 10 min before the end of experimental time. **P<0.01; ***P<0.001.

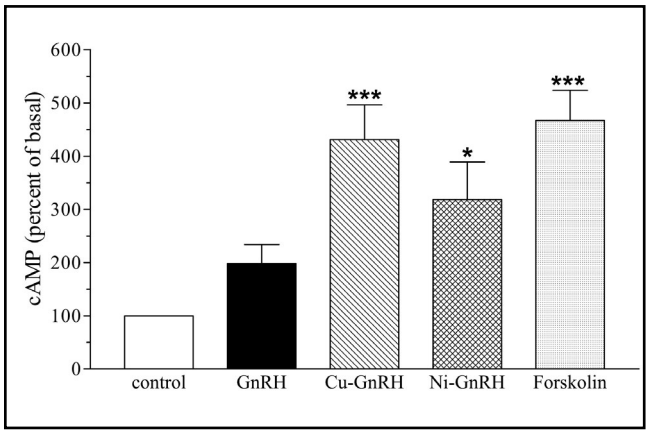


**Figure 4:** The effect of GnRH, Cu-GnRH and Ni-GnRH on intracellular cAMP accumulation after one hour incubation of porcine anterior pituitary cells. Treatment concentrations were $10^{-7}$ M. Forskolin (10 μM; used as a positive control) was added 10 min before the end of incubation. *P<0.05, ***P<0.001.

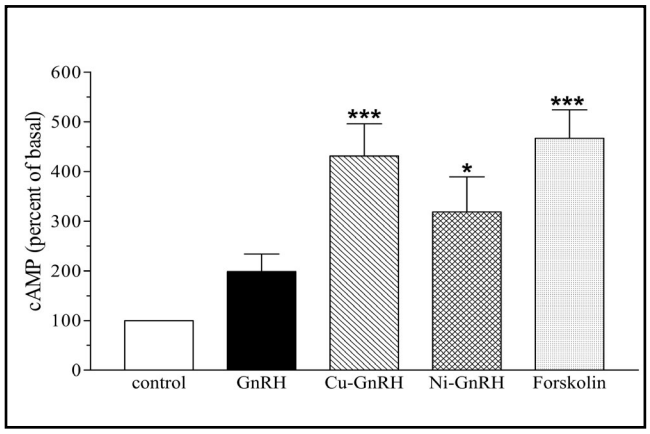


**Figure 5:** The effect of GnRH, Cu-GnRH and Ni-GnRH on intracellular accumulation of inositol phosphates in the porcine pituitary cells after 30 min of incubation. Treatment concentrations were $10^{-7}$ M. *P<0.05.

cAMP production by pituitary cells (431% of the control value; P<0.001). Its action was comparable with forskolin (467% of the control value; P<0.001) despite the fact that forskolin was added as late as 10 min before the end of treatment. In the presence of the Ni-GnRH complex, concentration of cyclic AMP in cells increased three times (318% of the control value; P<0.05). The effect of native GnRH on cAMP accumulation in pituitary cells was not significant.

### *Effect of GnRH, Cu-GnRH, and Ni-GnRH on Phosphoinositide Hydrolysis*

As it is shown in fig. 5, increased accumulation of inositol phosphates in pituitary cells was observed after 30 min of incubation only in the case of native GnRH (163% of the control value; P<0.05). Cu-GnRH and Ni-GnRH did not increase IPs output (130 and 150% of the control value, respectively).

## Discussion

Results of our earlier studies [9, 15–16] demonstrated, that it is possible to obtain stable complexes of copper, nickel and zinc with GnRH, although the stability of these complexes differs. In female rats, the GnRH complexes with copper, nickel and zinc were more potent stimulators of LH and FSH release than the native GnRH [16]. These complexes bound to GnRH receptor in a competitive way with buserelin [9, 15]. The molecular structure of the GnRH complexes with copper and nickel was determined in our previous study [9].

We have demonstrated that the copper and nickel complexes with GnRH stimulated LH and FSH release from the anterior pituitary [15–16]. Moreover, the role of copper in the GnRH release from the hypothalamus was described by Barnea and co-workers [1–5]. The mechanism of action of these complexes, however, is not known. Copper is present in hypothalamus and copper ions are necessary for amidation of glycine 10 in GnRH molecule what suggests a possibility of GnRH release to portal circulation partially in a complexed form. Direct participation of copper in release of LH from the pituitary was studied by Hazum [13] who proved that Cu acts directly on the pituitary.

In the present experiment, both native GnRH and the Cu and Ni complexes caused LH release into medium. However, only Cu-GnRH exhibited a clear dose-dependent effect. On the other hand, results of these experiments and those of our previous studies [9, 15–16] suggest that metal complexes with GnRH could affect the intracellular signaling pathways in the pig pituitary *in vitro***.** One hour incubation of pituitary cells with the copper- or nickel-GnRH complexes increased significantly the intracellular cAMP synthesis which was confirmed by a similar elevation of the extracellular cAMP level during 180 min incubation. The native GnRH did not increase cAMP in both examined time points. The intracellular synthesis of phosphoinositols was not affected by the GnRH complexes with copper and nickel, while GnRH alone induced inositol phosphates accumulation. This is in agreement with results of previous studies which indicated that GnRH stimulates phospholipid turnover in the anterior pituitary [21–23].

A role of cAMP in GnRH signaling in pituitary cells is not clear. It has been previously reported that GnRH stimulates adenylate cyclase activity in the pituitary gland [10, 17] and that GnRH antagonists inhibit cAMP accumulation [6, 14]. On the other hand, it was indicated that the acute release of LH in response to GnRH does not require the production or involvement of cAMP [8] and/or cAMP does not have an intermediate role in GnRH-induced gonadotropin release [20, 24]. However, Garrel et al. [12] described a mechanism of cross-talk between protein kinase A and protein kinase C pathways in gonadotropes. It is of interest that copper and nickel complexes with GnRH stimulate protein kinase A activity and cAMP synthesis in pituitary cells. The precise explanation of this phenomenon needs further research. We conclude that the Cu-GnRH and Ni-GnRH complexes increase LH release in the porcine pituitary cells although their intracellular signaling is different from that of the native GnRH. It seems that

metal complexes with GnRH deserve more attention in further studies.

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