Vol. 136, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1986 Pages 417-425

COPPER INDUCES LUTEINIZING HORMONE RELEASE AND DESENSITIZATION OF PITUITARY GONADOTROPES

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Received February 26, 1986

Copper stimulated LH release from cultured rat pituitary cells in a dose- and time-dependent manner. After 4 h of incubation with 10 μ M Cu^{2+}, LH release was stimulated by 3-fold. The release of LH stimulated by Cu^{2+} was Ca^{2+} dependent, thus excluding the possibility that the releasing activity of this divalent cation was due to a toxic effect on pituitary cells. The stimulatory action of Cu^{2+} is substantially mediated via the GnRH-receptors since Cu^{2+} inhibited ^{125}I-Buserelin binding and since a GnRH-antagonist blocked most of the Cu^{2+}-stimulated LH release (80%). Both GnRH (1 μM) and Cu^{2+} (10 μM) induced desensitization of pituitary cells to a subsequent stimulation of either GnRH (0.5 nM) or Cu^{2+} (10 μM). However, in contrast to GnRH, Cu^{2+} did not induce down regulation of GnRH receptors. These findings suggest that the Cu^{2+} effects are mainly mediated through the GnRH receptors.

Cations play an important role in the stimulation of pituitary luteinizing hormone (LH) release. Previous studies have shown that copper induces the release of various adenohypophysial hormones from bovine pituitaries (1). In addition copper can induce GnRH release from the hypothalamus into the hypophysial portal blood system, since isolated hypothalamic granules release GnRH in response to copper (2-4). Thus, the ability of copper to release gonadotropins and GnRH may explain the phenomenon that systemic administration of copper to female rabbits leads to ovulation (5).

Due to the fact that some cations stimulate LH release, the question arises whether they can also induce desensitization. It is well established that prolonged exposure of pituitary cells to GnRH induces desensitization of these cells to further stimulation by the same hormone (6-10). In addition, high concentrations of potassium can also induce desensitization of pituitary cells to GnRH, although to a lower extent than the hormone itself (11).

Recently, we have reported that copper can inhibit the binding of GnRH to rat pituitary membrane preparations and can induce LH release from whole rat

0006-291X/86 $1.50

pituitaries (12). In the present study, we have examined the mechanism by which copper induces LH release and desensitization of cultured rat pituitary cells.

MATERIALS AND METHODS

Materials

Collagenase, hyaluronidase and trypsin were purchased from Worthington Biochemical Co., Freehold, NJ; DNAse and bovine serum albumin from Sigma Chemical Co., St. Louis, MO; horse serum and antibiotic antimycotic solution from Bio-Lab, Jerusalem, Israel; minimum essential medium for suspension (S-MEM) and medium 199 were purchased from Grand Island Biological Co., Grand Island, N.Y.; [D-Ser(t-Bu)^{6},des-Gly^{10},ethylamide]GnRH (Buserelin) was kindly supplied by Dr. J. Sandow, Hoechst, Frankfurt; [D-pGlu^{1},D-Phe^{2},D-Trp^{3,6}]-GnRH (GnRh-antagonist) was obtained from Peninsula Laboratories Inc., San-Carlos, California.

Cell culture

Cell cultures were prepared as described earlier (13). Briefly, pituitaries of 3-month-old Wistar derived female rats (housed in our departmental colony) were minced, transferred to a spinner flask containing 0.05% trypsin in S-MEM and incubated for 45 min at 37°C. The medium was then replaced by fresh S-MEM containing 0.1% collagenase, 0.075% hyaluronidase and 0.1% bovine serum albumin (BSA), and the incubation was continued for another 30 min. DNAse (10 µg/ml) was present during all steps of dispersion to avoid clumping. Cells were washed with medium 199 containing 0.1% BSA and filtered through 4 layers of gauze to remove tissue fragments. After centrifugation (10 min, 800 x g), the cell pellet was resuspended in medium 199 containing 10% horse serum, 0.1% BSA and antibiotic antimycotic solution (4 µl/ml) and finally placed in tissue culture plates. Approximately 1.4x10^{6} cells were added to each 35 mm plastic culture dish and were maintained for 48 h.

Iodination of GnRH-analog

Buserelin ([D-Ser(t-Bu)^{6},des-Gly^{10}, ethylamide]GnRH) was iodinated by the lactoperoxidase method, applied to a column of Sephadex G-25 (4cm x 35 cm) previously equilibrated and eluted with 0.01 M acetic acid (14). The specific activity of the labeled peptides was about 1 mCi/µg.

Binding assay

After 48 h in culture, pituitary cells were washed with phosphate buffered saline (PBS) and removed from the culture dishes by incubation with PBS containing 2 mM EDTA (15 min at 37°C). Residual cells were scraped by a rubber policeman. Pituitary cells (0.5x10^{6} cells) were incubated with iodinated labeled Buserelin (60,000 cpm) and various concentrations of Cu^{2+} (cupric sulphate) in a total volume of 0.5 ml of Krebs Ringer Bicarbonate containing 1 mg/ml glucose (KRBG) and 0.1% BSA. After 90 min at 4°C, the binding reaction was terminated by rapid filtration through Whatman GF/C filters. Specific binding represents the bound radioactivity that can be competed for by 10^{-7}M unlabeled Buserelin.

LH release

After 48 h in culture, the cells were washed with KRBG and incubated with copper (usually 10^{-5}M) or GnRH (0.5 nM) for an additional 4 h, unless otherwise indicated. At the end of the incubation period, aliquots of the incubation solution were taken for LH determination.

Desensitization of pituitary cells

Cultured pituitary cells were washed with KRBG and subsequently incubated with copper (10 μM) or GnRH (1 μM). After 2 h, the cells were washed twice with KRBG (over a period of 30 min) and incubated in the presence or absence of copper (10 μM) or GnRH (0.5 nM). After 4 h at 37°C, LH release was determined by radioimmunoassay.

Radioimmunoassay (RIA) of LH

The amount of LH was assayed by radioimmunoassay using a kit supplied by the NIADDK, Rat Pituitary Hormone Program, NIH, Bethesda, MD. Results are expressed in terms of the RP-1 Rat Reference Preparations, as μg/ml of LH.

Statistical analysis

Data for Fig. 4 was analyzed by Student Neumann Keuls analysis (multiple range test). The other figures were analyzed by student's t test.

RESULTS

The ability of cupric ions to stimulate LH release from pituitary cells is shown in Fig. 1. Cultured pituitary cells were incubated with different concentrations of Cu^{2+} for 4 h at 37°C, and at the end of the incubation time the amount of LH released to the medium was determined. While 0.1 μM to 5 μM Cu^{2+} did not cause any significant release of LH, Cu^{2+} at a concentration range of 10 to 50 μM stimulated basal release by 3-fold. This stimulation was 30-40% of that obtained by 0.5 nM GnRH (Fig. 1). Under these experimental condi-

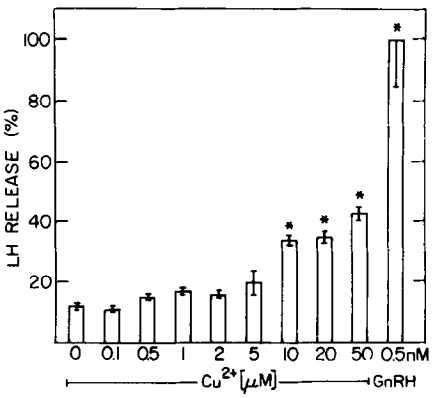


Fig. 1. Dose-dependent effect of cupric ions on LH release from pituitary cells. Pituitary cells, cultured for 48 h, were washed with Krebs Ringer Bicarbonate containing 1 mg/ml glucose (KRBG), and incubated with 0.5 nM GnRH or various concentrations of Cu^{2+} in KRBG. After 4 h at 37°C, aliquots of the medium were taken for radioimmunoassay of LH, as described under "Materials and Methods". Results are the mean + S.E. of 11-18 replicates from 3 separate experiments. *, $p<0.\overline{001}$ vs. basal release. 100% is equivalent to 6.0 μg/ml of LH release.

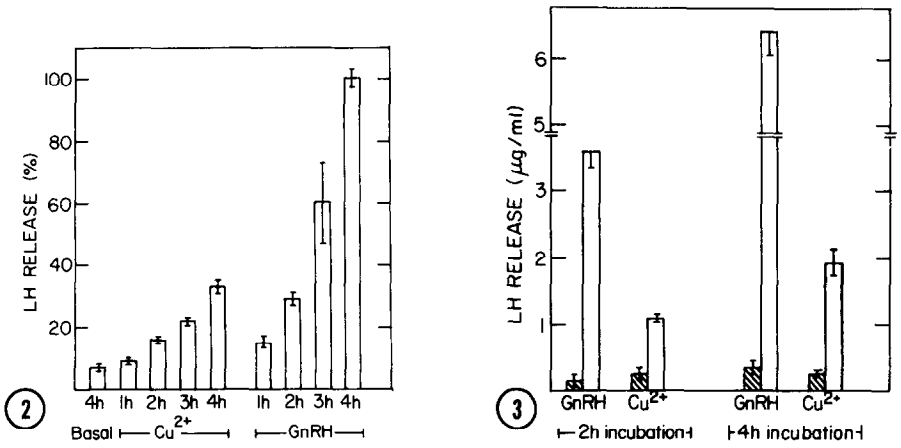


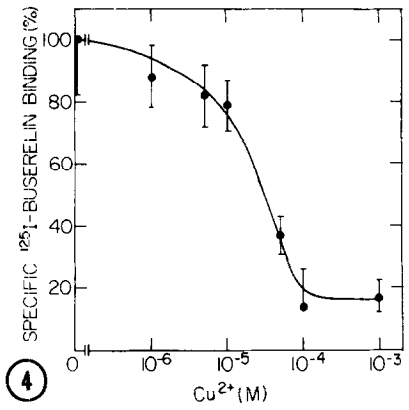
Fig. 2. Time-dependent effect of Cu^{2+} and GnRH on LH release from pituitary cells. Pituitary cultures were incubated with either 10 μM Cu^{2+} or 0.5 nM GnRH for various time periods. At the time indicated, the amount of LH released to the medium was assessed as described in the legend to Fig. 1. Results are the mean ± S.E. of 6 replicates from 2 different experiments.

Fig. 3. Effect of extracellular Ca^{2+} on LH release stimulated by Cu^{2+} or GnRH. Cells cultured for 48 h were incubated with 10 μM Cu^{2+} or 0.5 nM GnRH in the presence (□) or absence (▨) of Ca^{2+} (Krebs Ringer Bicarbonate without Ca^{2+}). After 2 or 4 h of incubation, aliquots of the medium were assayed for LH as described in the legend to Fig. 1. Results are the mean ± S.E. of 3-6 replicates.

tions, Cu^{2+}-treated cells had the same morphological appearance as untreated cells (data not shown).

Copper-stimulated LH release from pituitary cells was a time-dependent process (Fig. 2). Pituitary cells were incubated with either 10^{-5}M Cu^{2+} or 0.5 nM GnRH for various time periods and the amount of LH released to the medium was determined. After 1 h of incubation with Cu^{2+} the release of LH was not significantly higher than that of basal release. Longer incubation periods with Cu^{2+} caused enhancement of the release and after 4 h of incubation, Cu^{2+}-stimulated LH release was maximal, i.e. 3-fold than basal release. At all the incubation periods measured, GnRH-stimulated LH release was significantly higher than that of Cu^{2+}-stimulated LH release (Fig. 2).

Fig. 3 shows that LH release stimulated by Cu^{2+} is dependent on extracellular Ca^{2+}. Pituitary cells were incubated with either 0.5 nM GnRH or 10 μM Cu^{2+} in the presence or absence of Ca^{2+}. Cu^{2+}-stimulated LH release was significantly higher in the presence of Ca^{2+} than in the absence of this divalent



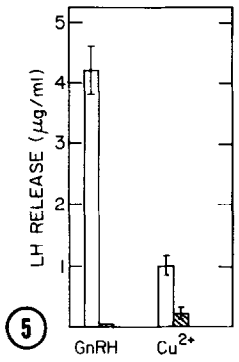


Fig. 4. Dose-dependent effect of Cu^{2+} on ^{125}I-Buserelin binding to pituitary cells. After 48 h, the cells were removed from the culture dishes and incubated ($0.5x10^6$ cells/tube) with ^{125}I-buserelin (60,000 cpm) and various concentrations of Cu^{2+} in 0.5 ml KRBG containing 0.1% BSA. The incubation was continued for 90 min at 4°C and the specific binding was measured as described under "Materials and Methods". Points are the mean ± S.E. of 6 replicates from 2 different experiments.

Fig. 5. Effect of a GnRH antagonist on LH release stimulated by Cu^{2+} or GnRH. Cultured pituitary cells were incubated in the presence (▨) or absence (□) of a GnRH antagonist for 1 h at 37°C. At the end of the preincubation time, GnRH (0.5 nM) or Cu^{2+} (10 μM) were added for an additional 4 h. Aliquots of the medium were then taken for RIA as described in the legend to Fig. 1. Results are the mean ± S.E. of 6-12 replicates for 2 different experiments.

cation, both after 2 or 4 h of incubation. This dependency on extracellular Ca^{2+} was also observed in the case of GnRH-stimulated LH release.

To analyze whether the stimulation of LH release by Cu^{2+} is mediated by GnRH receptors, the effect of cupric ions on the binding of ^{125}I-buserelin to pituitary cells was next examined (Fig. 4). Pituitary cells were removed from the culture dishes and binding assay was conducted in the presence of various concentrations of copper. While 1 μM to 10 μM Cu^{2+} did not affect ^{125}I-buserelin specific binding, 0.05 to 1 mM Cu^{2+} inhibited it significantly ($p<0.05$ by Student Neumann Keul's analysis). Under these experimental conditions the apparent IC_{50} value of Cu^{2+} was $3x10^{-5}$M.

To further establish that Cu^{2+}-stimulated LH release is mediated by GnRH-receptors we analyzed this effect in the presence of a GnRH antagonist [D-pGlu^1,D-Phe^2,D-Trp^{3,6}]-GnRH (Fig. 5). Cultured pituitary cells were preincubated with 0.5 μM GnRH-antagonist for 1 h at 37°C and subsequently either

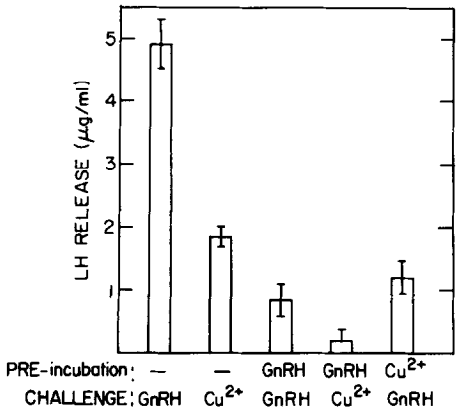


Fig. 6. Induction of pituitary cell desensitization by Cu^{2+} or GnRH. Pituitary cultures were washed with KRBG and incubated for 2 h at 37°C in KRBG without any additions, with 1 μM GnRH or with 10 μM Cu^{2+}. At the end of the incubation time, the cells were washed extensively and rechallenged with either 10 μM Cu^{2+} or 0.5 nM GnRH for an additional 4 h at 37°C. LH determination was conducted as described under "Materials and Methods". Results are the mean ± S.E. of 12-16 determinations from 2 different experiments.

0.5 nM GnRH or 10 μM Cu^{2+} were added. After an additional 4 h of incubation, the amount of LH released to the medium was determined. As shown in Fig. 5, preincubation with the GnRH-antagonist completely abolished GnRH-stimulated LH release, whereas LH release stimulated by Cu^{2+} was inhibited to a lower extent (80%).

We next examined whether the LH-releasing potency of Cu^{2+} can desensitize LH secretion from pituitary cells (Fig. 6). Cultured cells were preincubated with either 10 μM Cu^{2+} or 1 μM GnRH for 2 h, washed extensively and challenged with either 0.5 nM GnRH or 10 μM Cu^{2+} for 4 h, as indicated in the figure. A third group of cells was desensitized by 1 μM GnRH and challenged with the same hormone (0.5 nM). GnRH stimulated the release of 4.9±0.4 μg/ml of LH, while 10 μM Cu^{2+} induced the release of about 30% of this amount. Nevertheless, cells desensitized to GnRH responded to the same extent to both challenges i.e., 0.5 nM GnRH or 10 μM Cu^{2+}. Under these experimental conditions, desensitization by GnRH is not due to LH depletion (data not shown). Moreover, cells desensitized by cupric ions were unable to respond to GnRH challenge and the amount of LH released to the medium was not significantly different from that released by GnRH in GnRH-desensitized cells.

To exclude the possibility that Cu^{2+}-induced desensitization of pituitary cells is due to down-regulation of GnRH receptors, the effect of copper on ^{125}I-Buserelin binding was next examined. Cultured pituitary cells were preincubated with 10 μM Cu^{2+} for 2 h at 37°C. At the end of the incubation time, the cells were washed extensively (to dissociate the bound copper), removed from the culture dishes and binding assay was conducted. The results indicated that there was no significant difference in GnRH receptor content between copper-treated and non-treated cells (3.3×10^5 receptors/gonadotrope, data not shown).

DISCUSSION

Copper stimulates the release of LH from rat pituitary cells in a time- and dose-dependent manner. After 4 h of incubation with 10 μM Cu^{2+}, LH release is stimulated by 3-fold. Under the same experimental conditions GnRH stimulates LH release to a much higher extent, i.e. by at least 10-fold. It is well established that low concentrations of copper (10 μM or less) catalyze many biological processes, whereas higher concentrations non selectively oxidize various membrane constituents, thus leading to membrane disintegration (15,16). Therefore, it was essential to prove that Cu^{2+}-stimulated LH release from rat pituitary cells is not due to a toxic effect. This possibility is ruled out since: (i) LH release stimulated by copper is Ca^{2+}-dependent; (ii) a GnRH antagonist blocks substantially copper stimulated LH release, and (iii) cells treated with Cu^{2+} have the same morphological appearance as untreated cells.

Recently, it has been shown that Cu^{2+} can inhibit [^{3}H]estradiol binding to cytoplasmic receptors of whole rat uteri and thereby interferes with hormone delivery to target cell nuclei (17). Previous studies have shown that various monovalent and divalent cations, i.e. Ag^+, Hg^{2+}, Cu^{2+} and Zn^{+2}, inhibit [^{3}H]progesterone binding to cytosolic preparations of human myometrium at concentrations close to 0.1 μM (18). Under our experimental conditions Cu^{2+} inhibits ^{125}I-buserelin binding, in a dose dependent manner, with an apparent IC_{50} value of 30 μM. This IC_{50} value is identical to that obtained in our

previous study carried out with pituitary membrane preparations (12). Although, Cu^{2+} specifically interacts with the GnRH receptors, its LH releasing activity cannot be solely attributed to this interaction for the following reasons: (i) a GnRH-antagonist does not completely block LH release stimulated by copper; (ii) Cu^{2+} stimulates the release of LH and TSH (unpublished data) (in a calcium dependent manner) as well as other adenohypophysial hormones (1); and (iii) Heavy metals, including copper, can increase Ca^{2+} permeability as has been shown in sarcoplasmic reticulum vesicles (19). Thus, a small portion of the LH releasing activity of Cu^{2+} is not GnRH-receptor mediated, but rather involves some other mechanisms.

To further elaborate on the Cu^{2+} effect, we either desensitized pituitary cells by incubating them with GnRH and then challenged with Cu^{2+} or vice versa. The results indicate that pituitary cells desensitized to GnRH, respond to a very limited extent to Cu^{2+}-challenge. In addition, preincubation of the cells with Cu^{2+} induces desensitization to further stimulation with GnRH. Under these experimental conditions, cupric ions cause neither down regulation nor irreversible modification of the GnRH receptors.

In a recent study we have shown that the structural integrity of the GnRH-receptors is preserved in desensitized cells (20), suggesting that major structural alterations are not associated with GnRH-induced desensitization. Previous studies have already shown that high concentrations of K^+ can induce desensitization of pituitary cells (11). Since K+ induce LH release by a different mechanism than GnRH, the data suggest that desensitization involves post-receptor mechanisms. Thus, it can be postulated that LH release and desensitization induced by Cu^{2+} are mainly mediated through the GnRH receptor.

ACKNOWLEDGMENTS

This work was supported by the Minerva Foundation, by the Fund for Basic Research administered by the Israel Academy of Sciences and Humanities and by the United States Israel Binational Science Foundation. We thank Mrs. M. Kopelowitz for typing the manuscript and Mrs. A. Levy for excellent technical assistance.

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