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COPPER AND THIOL REGULATION OF GONADOTROPIN RELEASING HORMONE BINDING AND LUTEINIZING HORMONE RELEASE

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The effects of copper, sulfhydryl and disulfide reagents on gonadotropin releasing hormone (GnRH) binding to pituitary membrane preparations and on luteinizing hormone (LH) release from pituitaries of immature female rats were studied. Copper ions reduced the specific binding of ^{125}I-labeled [D-Ser(t-Bu)^{6}, des-Gly^{10}-ethylamide]GnRH (Buserelin) to pituitary membranes in a dose responsive manner. Fifty percent inhibition of the specific binding was obtained at 3 x 10^{-5}M Cu^{+2}. The decreased binding stems from a 8-fold decrease in the apparent affinity of Buserelin, in the presence of copper ions. Cupric sulfate was examined for its ability to affect basal and GnRH stimulated LH release. Copper stimulated basal LH release 18-fold and 8-fold at 10^{-4}M and 10^{-5}M, respectively. The LH release in response to Cu^{+2} was calcium dependent. The effect of Cu^{+2} on GnRH stimulated LH release was a combined effect of Cu^{+2} on GnRH binding, GnRH on LH release and Cu^{+2} on LH release. N-ethylmaleimide, hydrogen peroxide and dithiothreitol did not alter significantly the specific binding of ^{125}I-labeled Buserelin. N-ethylmaleimide and hydrogen peroxide did not affect GnRH stimulated LH release, whereas dithiothreitol (1 mM) significantly inhibited GnRH stimulated LH release. The present findings may explain the induction of ovulation by copper and the reduction in serum LH after injection of reducing agents.

Gonadotropin releasing hormone (GnRH) is a hypothalamic decapeptide, which affects gonadotropin release by interacting with specific receptors on gonadotrope cells. Cations play an important role in the stimulation of pituitary luteinizing hormone (LH) release. Recent studies have indicated that calcium ions may actually perform the role of an intracellular mediator in this system (reviewed in ref. 1). In addition, LH release in response to cell depolarization by K^{+} is Ca^{+2}-dependent (2-4).

Several groups of investigators have observed that systemic administration of copper salts to female rabbits induces ovulation (5), probably by stimulating the release of GnRH from the hypothalamus (6,7). Recently, it has been shown that copper stimulates the release of GnRH from isolated hypothalamic granules (8). In contrast to the effects of copper, subcutaneous injection of cysteamine reduces serum LH concentrations in adult male rats without altering the hypothalamic GnRH content (9). In view of these findings, we investigated the effects of copper, dithiothreitol and other sulfhydryl rea-

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gents on the binding of GnRH to rat pituitary membranes and LH release from pituitaries of immature female rats.

MATERIALS AND METHODS

Iodination and pituitary membrane preparations.

[D-Ser(t-Bu)^{6},des-Gly^{10},ethylamide]GnRH (Buserelin), kindly supplied by Dr. J. Sandow, Hoechst, Frankfurt) was iodinated by the lactoperoxidase method as described previously (10). The specific activity of the labeled peptide was approximately 1000 μCi/μg.

Pituitary membranes were prepared from Wistar-derived female rats as previously described (10). Briefly, the glands were homogenized gently with a Dounce homogenizer at 4°C in assay buffer (10 mM Tris.HCl, pH-7.4, 0.1% BSA) containing 1.0 mM dithiothreitol and centrifuged for 10 min at 1000 x g. The supernatant was then centrifuged for 20 min at 20,000 x g. The pellet was resuspended in the same buffer, centrifuged at 20,000 x g for 20 min and finally suspended in assay buffer.

Binding assays

The labeled peptide (40,000 cpm) was incubated with 20 to 30 μg protein of pituitary membranes in a total volume of 0.5 ml of assay buffer for 90 min at 4°C. The homogenate was then filtered under vacuum through Whatman GF/C filters presoaked in 0.1% of BSA, washed with 10 ml of ice-cold buffer and the filters counted in a gamma counter. Specific binding (80% of total binding) represents the bound radioactivity which can be displaced by prior addition of 10^{-6}M unlabeled Buserelin. Each value is the mean of triplicate incubations, which diverged by less than 7%.

Bioassay

Pituitaries derived from 12-day-old female rats were incubated in Krebs Ringer Bicarbonate containing 10^{-9}M GnRH or a combination of 10^{-9}M GnRH and the tested compounds. The incubation procedure has been described in detail elsewhere (11). At the end of the incubation period, aliquots of the medium were analyzed by radioimmunoassay (RIA) for LH content using the kit kindly supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), Rat Pituitary Program. Results are expressed in terms of the RP-1 reference preparation.

RESULTS

The effect of cupric ions on the binding of ^{125}I-labeled Buserelin to pituitary membrane preparations is shown in Fig. 1. Incubations of the membrane with cupric sulfate resulted in a dose-dependent decrease of the specific binding. Fifty percent inhibition of the specific binding was obtained at 3×10^{-5}M Cu^{+2}. This decrease is only a loss of specific binding, because there was no significant change in non-specific binding. Preincubation of pituitary membranes with 10^{-4}M Cu^{+2} and subsequent extensive washing resulted in the restoration of GnRH binding suggesting that the Cu^{+2} effect is reversible (data not shown). The inhibitory effects with Cu^{+2} are not specific for this ion because inhibition of receptor binding is also seen with Na^{+}, K^{+}, Mg^{+2}, Ca^{+2}, Mn^{+2}, Co^{+2} and La^{+3} (1,10,12,13), although higher concentrations are required.

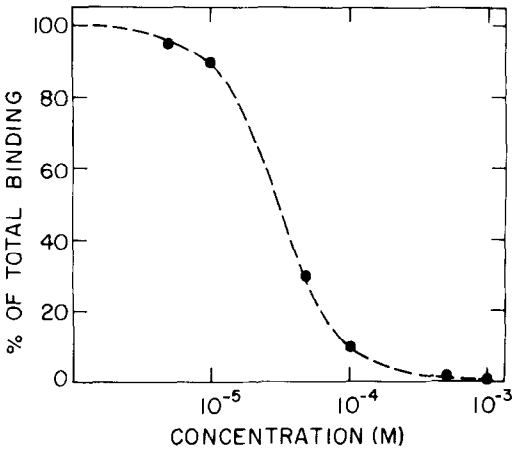

Fig. 1. Effect of Cu^{+2} on the binding of ^{125}I-labeled Buserelin to pituitary membrane preparations. The radioactive Buserelin (40,000 cpm) was incubated with different concentrations of Cu^{+2} for 90 min at 4°C in a final volume of 0.5 ml containing pituitary membranes (20 to 30 µg of protein/ml) and the binding was measured as described in Materials and Methods.

Fig. 2 shows the effect of 5 x 10^{-5}M Cu^{+2} on the competition of binding of ^{125}I-labeled Buserelin by unlabeled Buserelin. Incubation of the membrane with cupric sulfate resulted in a decrease of the specific binding. Scatchard analysis (Fig. 2, inset) of receptor binding in the presence of Cu^{+2} revealed a decrease in receptor affinity (1.2 nM) compared to binding without added Cu^{+2} (0.15 nM). Thus, there was a 8-fold decrease in receptor affinity without a significant change in receptor number.

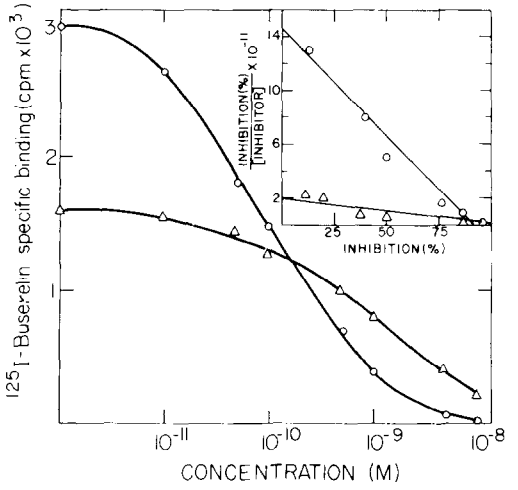

Fig. 2. Competition of binding of ^{125}I-labeled Buserelin binding by unlabeled Buserelin in the presence (Δ—Δ) and absence (O—O) of 5 x 10^{-5}M Cu^{+2}; inset, Scatchard plot. Specific binding was measured as described in Materials and Methods. Values are the mean of triplicate tubes of four separate experiments.

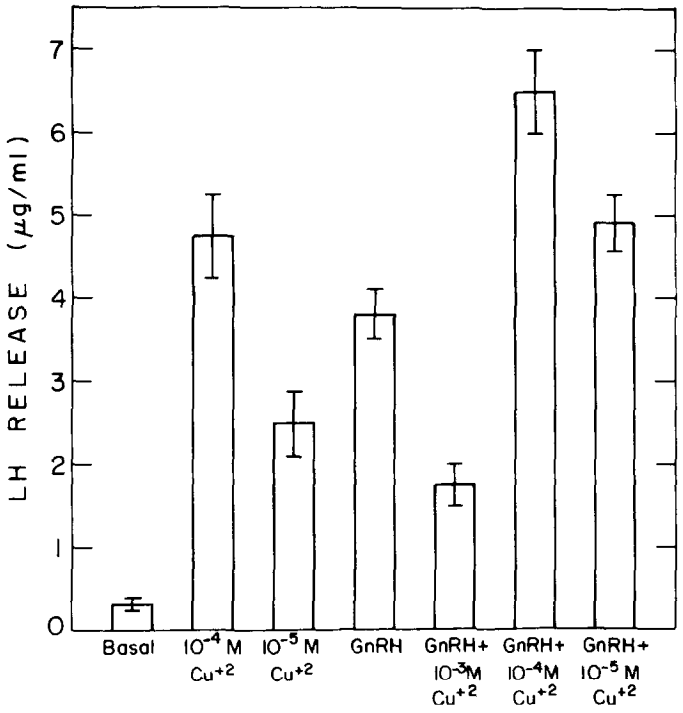

Fig. 3. Effect of Cu^{+2} on basal and GnRH (1 ng/ml) stimulated LH release from pituitaries of immature female rats. The pituitaries were incubated as described in Materials and Methods. Each bar represents mean ± S.E. of 12 determinations in two separate experiments.

Cupric sulfate was examined for its ability to affect basal and GnRH stimulated LH release. As shown in Fig. 3, basal LH release was 0.3 ± 0.05 μg/ml whereas Cu^{+2} at concentrations of 10^{-4}M and 10^{-5}M elicited 4.75 ± 0.5 and 2.5 ± 0.4 μg/ml of LH, respectively. Thus, Cu^{+2} stimulated basal LH release eighteenfold and eightfold at 10^{-4}M and 10^{-5}M, respectively. The effect of Cu^{+2} on GnRH stimulated LH release was a combined effect of Cu^{+2} on GnRH binding (Fig. 1), GnRH on LH release and Cu^{+2} on LH release (Fig. 3). Thus, a combination of 1 ng/ml GnRH and 10^{-3}M Cu^{+2} elicited only 1.75 ± 0.25 μg/ml of LH, because at this concentration Cu^{+2} abolished all the specific binding of GnRH (Fig. 1) and had very low releasing activity by itself (Fig. 4). At 10^{-4}M and 10^{-5}M Cu^{+2}, 90% and 10% of the specific binding of GnRH was inhibited respectively (Fig. 1). Thus, the combined effect of GnRH and Cu^{+2} at 10^{-4}M or 10^{-5}M on LH release was the additive effect of Cu^{+2} and the residual activity of GnRH. Cupric ions at the concentrations examined did not affect at all the binding of rat LH to the antibodies against rat LH.

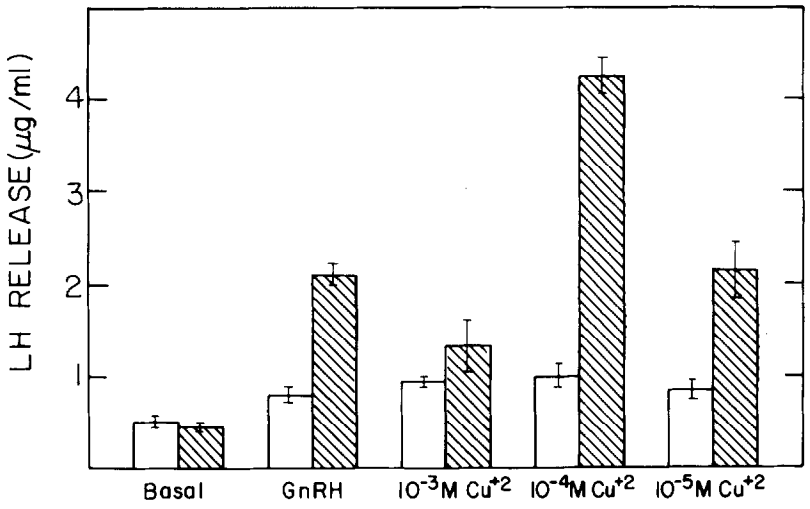

Fig. 4. Effect of Cu^{+2} and GnRH (0.5 ng/ml) on LH release in the presence (▨) and absence (□) of Ca^{+2} (Krebs Ringer Bicarbonate without Ca^{+2}). For experimental details see the legend to Fig. 3.

The effect of extracellular calcium on Cu^{+2} and GnRH stimulated LH release is shown in Fig. 4. Pituitaries of immature female rats incubated in medium containing Ca^{+2} were responsive to stimulation by both GnRH and Cu^{+2}, whereas in Ca^{+2}-free medium they were unresponsive. This suggests that LH release in response to both GnRH and Cu^{+2} is Ca^{+2} dependent.

The effects of sulfhydryl and disulfide reagent on ^{125}I-labeled Buserelin binding and GnRH stimulated LH release are summarized in Table 1. N-ethylmaleimide, hydrogen peroxide and dithiothreitol did not alter significantly the binding. N-ethylmaleimide and hydrogen peroxide did not affect GnRH stimulated LH release, whereas dithiothreitol at a concentration of 1 mM significantly inhibited GnRH stimulated LH release (Table 1).

DISCUSSION

The present study demonstrates that: (i) Cupric ions reduce, in a dose related manner, the binding of GnRH to its receptor by decreasing the apparent affinity; (ii) Cu^{+2} stimulates basal LH release through a calcium dependent mechanism, and (iii) dithiothreithol inhibits GnRH stimulated LH release without altering receptor binding. Similar to these findings, studies with opiate receptors have indicated that cupric ions cause naloxone-reversible analgesia when injected intracerebroventicularly in mice, whereas dithiothreitol, reverses the analgesia induced by cupric ions and antagonizes

Table 1. Effect of sulfhydryl and disulfide reagents on ^{125}I-labeled Buserelin binding and GnRH stimulated LH release.

Reagent[a] (mM)	Specific ^{125}I-Buserelin binding (% of control)[b]	LH release[c] µg/ml
N-Ethylmaleimide		
0.01	103	3.9±0.6
0.1	95	4.3±0.5
1	99	4.1±0.4
Hydrogen peroxide		
0.01	95	3.2±0.5
0.1	92	3.5±0.4
1	93	3.6±0.7
Dithiothreitol		
0.01	101	3.7±0.3
0.1	94	3.2±0.4
1	92	1.9±0.2

[a] All reagents were dissolved immediately before use.
[b] Specific binding was determined as described in Materials and Methods. Each value is the mean of triplicate incubations, which varied by less than 7%.
[c] GnRH at 1 ng/ml elicited 3.75±0.3 µg/ml LH release. The values indicated are the combined effects of 1 ng/ml GnRH and the tested compounds. The pituitaries were incubated as described in Materials and Methods. Each value represents mean ± S.E. of 12 determinations in two separate experiments.

analgesia induced by morphine (14). In vitro, Cu^{+2} abolishes opiate receptor binding (15) and dithiothreitol does not significantly affect the binding of enkephalin to neuroblastoma cells (16).

Cupric ions play an important role in mammalian reproduction. It has been observed that copper deficiency leads to infertility in guinea pigs and rats (17), whereas administration of copper to female rabbits induces ovulation (5). These effects have been attributed to the release of GnRH from the hypothalamus (6,7), and recently it has been shown that CuATP stimulates the release of GnRH from isolated hypothalamic granules (8). The findings presented in this study strongly indicate that cupric ions have a direct effect on the pituitary which results in an enormous release of LH. This release may explain the induction of ovulation by copper. Nevertheless, it is possible that Cu^{+2} may affect both LH release from the pituitary and GnRH release from the hypothalamus.

Recently, it has been reported (9) that cysteamine injections reduce concentrations of pituitary and serum gonadotropin in adult male rats, with no significant reductions in radioimmunoassayable hypothalamic GnRH. In addition, the results of employing cysteamine and GnRH indicate that the pituitaries of such rats are less responsive to GnRH. In the present study another reducing reagent, dithiothreitol, was tested in vitro, using pituitaries from immature female rats. The results indicate that dithioth-

reitol inhibits GnRH stimulated LH release without altering binding capacity. Other sulfhydryl blocking-reagents such as N-ethylmaleimide and hydrogen peroxide have no effect on GnRH binding and have a small effect on GnRH stimulated LH release. This suggests that a mechanism of oxidation-reduction of thiols (14) is probably not involved in modulating GnRH receptor function.

Calcium fulfils the requirements of an intracellular mediator for GnRH-stimulated LH release from the pituitary (reviewed in ref. 1). As suggested by Conn et al. (1), GnRH action may be divided into three sequential steps: 1) interaction of GnRH with the receptors; 2) mobilization of ionic calcium, and 3) expulsion of the contents of the gonadotropin secretory granule to the extracellular space. Moreover, the requirement for Ca^{+2} in GnRH-stimulated LH release is not mediated through a specific action of this ion at the level of receptor binding, but occurs at a post-receptor locus. The finding that LH release in response to Cu^{+2} is calcium dependent (no release in the absence of extracellular calcium) suggests that cupric ions act by increasing, or mobilizing, intracellular calcium ions that evoke LH release.

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