

Release of chicken luteinising hormone-releasing hormone-I (cLHRH-I) by mediobasal hypothalamus in the cockerel: validation of an incubation system and effect of excitatory amino acids

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Summary — An *in vitro* system for the incubation of mediobasal hypothalami (MBH) of cockerels and a radioimmunoassay for chicken luteinising hormone-releasing hormone-I (cLHRH-I) were developed. The size of the hypothalamic fragment (MBH including the median eminence) and the incubation conditions used (40°C, under constant shaking and gassing) preserved the physiological properties of the tissue. It was possible to maintain the MBH *in vitro* and to study the LHRH release for several hours. The assay proved sensitive enough ($ED_{50} = 0.794$ pmol/tube, *ie* 4.59 pg/ml) and sufficiently precise (within-assay coefficient of variation = 4.4% and between-assay coefficient of variation = 10.2%) to measure the amounts of peptide released in the incubation medium. The use of this incubation system provided the first evidence of the stimulating effect of the excitatory amino acids glutamate, NMDA and kainate on the secretion of cLHRH-I in birds. Our results suggest that the effect on the NMDA receptor is predominant.

chicken LHRH / hypothalamus / incubation / excitatory amino acids

Résumé — Sécrétion de *chicken luteinising hormone-releasing hormone-I* (cLHRH-I) par l'hypothalamus médiobasal chez le coq : validation d'un système d'incubation et effet des acides aminés excitateurs. Un système d'incubation *in vitro* d'hypothalamus médiobasal (HMB) de coq et un dosage radioimmunologique de la chicken luteinising hormone-releasing hormone-I (cLHRH-I) ont été développés. La taille du fragment hypothalamique prélevé (HMB comprenant l'éminence médiane) et les conditions d'incubation utilisées (40°C, sous agitation et oxygénation constantes) respectent les propriétés physiologiques du tissu. Ils ont permis de le maintenir *in vitro*

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et d'étudier la sécrétion de cLHRH-I pendant plusieurs heures. Le dosage s'est révélé suffisamment sensible ($ED_{80} = 0,794$ pmole/tube, c'est-à-dire 4,59 pg/ml) et précis (coefficient de variation intradosage = 4,4% et coefficient de variation interdosages = 10,2%) pour mesurer de façon fiable les quantités de neuropeptide libérées dans le milieu d'incubation. L'utilisation de ce système a permis de mettre en évidence pour la première fois chez les oiseaux l'effet stimulateur des acides aminés excitateurs : glutamate, NMDA et kainate sur la sécrétion de cLHRH-I. Les résultats obtenus suggèrent un effet prédominant sur le récepteur NMDA.

poulet-LHRH / hypothalamus / incubation / acides aminés excitateurs

INTRODUCTION

The release of luteinising hormone-releasing hormone (LHRH) by hypothalamic neuron terminals at the level of the median eminence is one of the major regulating steps of reproductive function and the direct measurement of the secretion of LHRH is required for the understanding of reproductive physiology. Thus, several *in vivo* (Levine and Ramirez, 1986, in the rat; Caraty and Locatelli, 1988, in the ram) and *in vitro* (review in Wibullaksanakul and Handelsman, 1991) techniques have been developed. The *in vitro* approach has the advantage of allowing the direct testing of the effect of various substances on the secretion. Two types of *in vitro* methods have been described: superfusion (Gallardo and Ramirez, 1977, in the rat; Knight, 1983, in the chicken) and static incubation (Rotzstejn *et al*, 1976, in the rat; Katz *et al*, 1990, in the chicken). Wibullaksanakul and Handelsman (1991) compared both systems in a systematic study and concluded the superiority of static incubation, which they judge technically simpler, more robust and accurate than superfusion. With the aim of studying the regulation of LHRH secretion in the chicken, we have developed an incubation system for chicken mediobasal hypothalamus (MBH) and a radioimmunoassay sensitive enough for reliable measurement of the amounts of chicken LHRH-I released *in vitro*. Indeed, from the 2 forms of LHRH known in the chicken, chicken LHRH-I

(cLHRH-I) (King and Millar, 1982a,b) and chicken LHRH-II (cLHRH-II) (Miyamoto *et al*, 1984), only cLHRH-I has been shown to be released from the median eminence (Katz *et al*, 1990). Both techniques were validated. To validate the incubation technique, the effect of potassium and calcium on the *in vitro* secretion of cLHRH-I was studied. The system was then used to investigate the action of excitatory amino acids (EAAs) on LHRH release, not yet documented in birds.

MATERIALS AND METHODS

Animals

Adult cockerels (meat-type breeders, T55, SASSO) were housed in individual cages under a photoschedule of 16 L:8 D. Food was restricted to 100 g per d as recommended for meat-type breeders and water was available *ad libitum*.

Hypothalamic tissue

Birds were killed by cervical dislocation without anesthesia. Hypothalamic fragments including MBH and the median eminence were rapidly dissected using the posterior border of the supra-optic decussation as the anterior limit and the root of the oculomotor nerves as the posterior limit. The fragments dissected extended laterally 1 mm from the third ventricle. Such fragments exclusively contain the nerve terminals of cLHRH-I neurons. The cell-bodies of these neurons are

located in the preoptic and septal areas (Sterling and Sharp, 1982; Józsa and Mess, 1982). The cLHRH-II cell bodies are situated in the oculomotor complex of the midbrain and they do not seem to project to the median eminence and no fibres were detected in the hypothalamus (Mikami *et al*, 1988; Kuenzel and Blhäser, 1991). The MBH were maintained in oxygenated artificial avian cerebrospinal fluid (ACSF) on ice during collection. ACSF was prepared immediately prior to the experiment (for composition, see Knight, 1983). Bacitracin (300 mg/l) was added to inhibit the activity of endogenous peptidase.

Static incubation system

One MBH was placed in each glass tube containing 500 μ l ACSF. The tubes were incubated in a warmed (40°C) water-bath with shaking (50 cycles/min). Each tube was individually gassed with 95% O₂/5% CO₂ throughout the incubation. A preincubation period of 1 h was observed to allow stabilisation of the basal release of GnRH. The incubation medium was collected and replaced with 500 μ l fresh medium every 30 min without further washing of the tissue. Collected fractions were stored frozen at -20°C until cLHRH-I concentration was measured.

Production of antibody to cLHRH-I

Synthetic cLHRH ([Gln8]) GnRH, Peninsula Laboratories Ltd, St Helens, Merseyside, UK) was conjugated with human serum albumin (HSA) using the carbodiimide method (Fraser *et al*, 1974). Peptide (8 mg) was mixed with 91.6 mg HSA (Sigma) and 80 mg 3-(3-dimethylamino-propyl)carbodiimide (Sigma). After shaking for 7 h, the peptide-protein conjugate was separated by dialysis against 0.01% mercaptoethanol, with several changes, for 48 h.

Rabbits were immunized by intra-dermal injection with the cLHRH-I conjugate homogenized with complete Freund's adjuvant. Four successive injections were performed at 7-d intervals. An iv booster injection, using Phénergan® (promethazine) as anti-histaminic agent, was administered 4 weeks after the last injection and was followed by several booster injections at regular intervals. After a final booster injection, one

antiserum (516/13) proved suitable for measurement of cLHRH-I release *in vitro*.

Radioiodination of cLHRH-I

Synthetic cLHRH-I was labelled with ¹²⁵I using the iodogen method (Salacinski *et al*, 1979). Iodogen (5 μ g, evaporated to dryness in a polypropylene tube) was used with 5 μ g cLHRH-I diluted in 0.01 M PBS and 0.5 mCi [¹²⁵I]-Nal (IMS 30, Amersham, France). After an incubation of 15 min, the reaction was stopped by adding 100 μ l PBS. The labelled peptide was purified using a method adapted from Knight (1983). After a first separation of free iodine on Dowex-resin (1 x 8-400, Sigma), the final purification was performed on a 0.7 x 15 cm carboxymethylcellulose (Sigma) column equilibrated with 0.002 M ammonium acetate (pH 4.5). Fourteen fractions (2 ml) were eluted with 0.002 M ammonium acetate and 10 further fractions with 0.06 M ammonium acetate. The cLHRH-I fraction displaying the highest activity was used for the assays.

Radioimmunoassay of cLHRH-I

Dilute samples (0.01 M PBS, 0.025 M EDTA, 0.05% bovine serum albumin and 0.01% azide) were assayed in duplicate following an assay procedure adapted from Knight (1983). Samples were incubated 24 h at 4°C in the presence of antiserum 516/13 at the final dilution of 1/180 000 (B/T = 35%). The labelled hormone (10 000 cpm/tube) was added on the 2nd day and tubes were incubated 48 h at 4°C. On the 4th day, the sheep anti-rabbit immunoglobulin was added with normal rabbit serum and polyethylene glycol. After 45 min of incubation, the tubes were centrifuged, the supernatant aspirated and the radioactivity of the pellets counted with a gamma counter. The specificity, sensitivity and precision of the assay were determined.

Validation of the incubation system

The effect of depolarization on the release of cLHRH-I was studied on a group of 4 MBH. The depolarization was achieved by a 10-fold increase of the concentration of potassium from 6.4 to 64

mM. The resulting increase in cations was compensated by the decrease of the concentration of sodium (from 126.1 to 68.5 mM). Six 30-min fractions were collected with the potassium being increased in the 30–60 and 150–180 min fractions.

The dose–response relationship of the potassium-induced release of cLHRH-I was studied on 4 groups of 4 MBH. Two 30-min incubation periods were observed. The MBH were incubated with ACSF containing 6.4 mM potassium for the first 30 min and with 32 mM, 48 mM, 56 mM or 64 mM potassium for the second 30 min incubation period. In subsequent experiments the submaximal dose of 56 mM was used.

The effect of the absence of calcium in ACSF on the 56 mM potassium-induced release was studied on 2 further groups of 4 MBH. The basal release (6.4 mM potassium) was measured in the first incubation fraction. During the second period, the first group of MBH was incubated with 56 mM potassium ACSF containing 3 mM calcium, while the second group was incubated with 56 mM potassium without calcium. In this group, the calcium ions were replaced by magnesium ions and EDTA was added to ACSF.

Effect of EAA on the release of cLHRH-I in vitro

The release of cLHRH-I was evaluated in the presence of 100 μ M glutamate (GLU) and 100 μ M *N*-methyl-D-aspartate (NMDA). Two groups of 4 MBH were used. Two 30-min incubation periods were used. The basal release of cLHRH-I was determined during the first incubation period and the effect of the 2 EAA in the second.

The effect of increasing doses (1 μ M, 10 μ M, 100 μ M) of NMDA and kainic acid (KA) were studied. Two groups of 4 MBH were used. Fractions were collected every 30 min. The doses were applied at 1.5-h intervals. Each stimulation with EAA was preceded by a basal release fraction, and followed by a washing fraction which was not collected.

Statistical analysis

Within-treatment comparisons were performed by a paired *t* test and between treatment comparisons by a *t* test.

RESULTS

Validation of the radioimmunoassay for cLHRH-I

The study in our cLHRH-I radioimmunoassay system, using antiserum 516/13, of the immunoreactivity of increasing concentrations of several hypothalamic peptides revealed 100% cross-reaction with mammalian LHRH and 20% cross-reaction with chicken LHRH-II. No cross-reaction could be detected with leucine-enkephalin, methionin-enkephalin, thyrotropin-releasing hormone (TRH) and corticotropin-releasing factor (CRF) up to a concentration of 10 μ g/ml, *ie* between 1 and 10 μ M. The dose that inhibited the binding of the radiolabelled hormone by 50% (ED_{50}) was 4.85 pg/tube, *ie* a concentration of 24.25 pg/ml. The limit of sensitivity of the assay, expressed as the dose that inhibited the binding of the radiolabelled hormone by 20% (ED_{80}) was 0.916 pg/tube, *ie* 4.58 pg/ml. The immunoreactivity of serial dilutions of hypothalamic extracts from cockerels proved parallel to the standard curve. The intra- and inter-assay coefficients of variation were, 4.4 and 10.2%, respectively.

Validation of the incubation system

The depolarization induced by 64 mM potassium elicited an 8-fold increase ($P < 0.01$) in cLHRH-I release (fig 1). The second depolarization occurring 2 h later induced an equivalent increase ($P < 0.01$) in the secretion of the peptide. Experiment 2 showed that the depolarization-induced release of cLHRH-I was dose-dependent (fig 2a). Each concentration of potassium elicited a significant increase (compared with basal release) in the secretion ($P < 0.05$ for 32 mM and $P < 0.01$ for the other 3 concentrations). The omission of calcium ions in

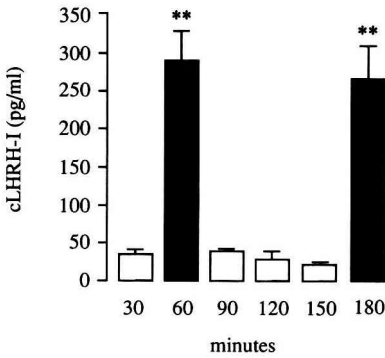


Fig 1. Effect of depolarization achieved by a 10-fold increase in the concentration of potassium ion on the release of cLHRH-I by incubated MBH in cockerels. The MBH were exposed to ACSF containing 64 mM potassium for 2 collection periods at 2-h intervals (white bars: 6.4 mM potassium; black bars: 64 mM potassium). Fractions were collected every 30 min. Values are mean \pm sem ($n = 4$). ** $P < 0.01$ compared with immediately preceding basal release.

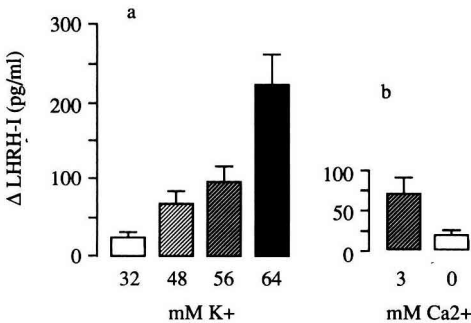


Fig 2. a) Effect of several concentrations of potassium on the release of cLHRH-I by incubated MBH in cockerels; **b)** effect of the absence of calcium ions in ACSF on the 56 mM potassium-induced release of cLHRH-I by incubated MBH in cockerels. Bars represent the mean increase in cLHRH-I concentration, i.e. the difference between the release elicited by potassium and the basal release measured in the immediately preceding fraction. Values are mean \pm sem ($n = 4$).

ACSF considerably reduced the 56 mM potassium-induced release of cLHRH-I. The 1.5-fold increase in the concentration observed in this group was not significant (fig 2b).

Effect of EAA on the release of cLHRH-I in vitro

Incubation of MBH in the presence of 100 μ M GLU and 100 μ M NMDA resulted in a 10-fold ($P < 0.001$) and a 4-fold ($P < 0.01$) increase in the release of cLHRH-I, respectively (fig 3).

NMDA elicited a dose-related increase in the secretion of cLHRH-I (fig 4a). The first significant ($P < 0.001$) increase was obtained with the 10 μ M dose, which multiplied the secretion by 4. The release elicited by the 100 μ M dose was very pronounced and was increased more than 10-fold. The stimulation of the release of cLHRH-I in the presence of KA also showed a dose-dependent profile (fig 4b). The response was, however, of much lesser amplitude than that with NMDA, and the highest dose used (100 μ M) only elicited a 2-fold increase in the secretion.

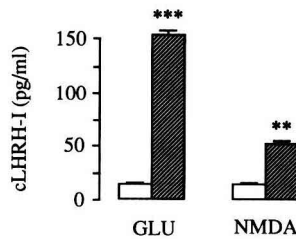


Fig 3. Effect of 100 μ M GLU and 100 μ M NMDA on the release of cLHRH-I by incubated MBH in cockerels (white bars: basal release; striped bars: release in the presence of GLU or NMDA). Values are mean \pm sem ($n = 4$). ** $P < 0.01$; *** $P < 0.001$ compared with basal release.

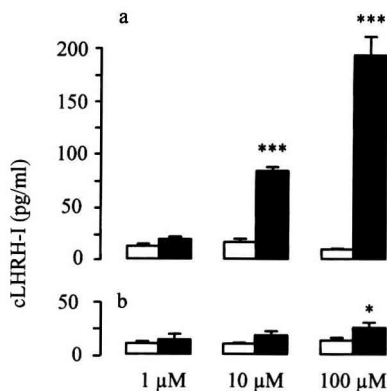


Fig 4. Effect of increasing doses of NMDA (a), and KA (b) on the release of cLHRH-I by incubated MBH in cockerels (white bars: basal release; black bars: release in the presence of the EAA). Values are mean \pm sem ($n = 4$). * $P < 0.05$; *** $P < 0.001$ compared with basal release).

DISCUSSION

As no release of cLHRH-II could be detected by MBH *in vitro* (Katz *et al*, 1990; and our preliminary work), the 20% cross-reactivity of our assay with cLHRH-II is not an obstacle to its use for *in vitro* experiments on chicken MBH. Furthermore, no cross-reactivity was found with the following other hypothalamic peptides: leu- and met-enkephalin, TRH and CRF. Moreover, the high sensitivity of this assay makes it suitable for the measurement of cLHRH-I *in vitro*.

The ability of incubated MBH to release cLHRH-I in response to a depolarization in a dose-dependent manner, and to respond to a second depolarization with a comparable magnitude, evidenced the good viability of the tissue in the incubation conditions used. Moreover, the necessity of the presence of calcium ions in the incubation medium for a response to occur showed that this release answers to the physiological mechanisms of neural secretion (Smith and Augustine, 1988).

Of the neurotransmitters known to exert an influence on the secretion of LHRH, EAAs have been shown to increase plasma concentrations of LH (Gay and Plant, 1987; Ondo *et al*, 1988) and agonists to stimulate the release of LHRH by hypothalamic tissue of rats *in vitro* (Bourguignon *et al*, 1989; Lopez *et al*, 1992). The use of EAAs in our incubation system allowed us to give first evidence of a similar effect on the secretion of cLHRH-I in the cockerel. The endogenous amino acid, GLU, stimulated the secretion significantly, and proved the most powerful of the 3 agonists that we used. The response elicited was 3 times that elicited by NMDA for the same dose. The effects of the 2 agonists NMDA and KA proved to be dose related, but the effect on KA receptors was shown to be limited. Our results suggest that the action on the NMDA receptor is predominant. Indeed the 10 μ M dose quadrupled the secretion of cLHRH-I for NMDA, while the maximum dose employed (100 μ M) only doubled the secretion for KA. This finding is in agreement with that of Bourguignon *et al* (1989) that NMDA is more potent than KA in eliciting the release of GnRH by incubated hypothalimi in the rat. However, the specificity of these effects in the chicken has to be further documented by the use of antagonists.

In conclusion, our static incubation system coupled to the radioimmunoassay proved to be suitable for the study of cLHRH-I release by cockerel MBH. We were thus able to bring first evidence of the stimulatory action of EAAs on the secretion of cLHRH-I in the cockerel and hence of their possible involvement in the control of reproduction through modulation of LH release in this species.

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