INFLUENCE OF CORTICOTROPIN-RELEASING FACTOR ON THE *IN VITRO* THYROXINE AND THYROTROPIN SECRETION IN NEWLY HATCHED FOWL

by

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SUMMARY

Injections of ovine corticotropin-releasing factor (oCRF) are known to increase circulating thyroid hormone levels in the chicken embryo (Meeuwis *et al.*, 1989). This can in part be explained by a direct effect of the hypothalamic hormone on the thyroid gland and/or the stimulation of the thyrotropin (TSH) release from the pituitary. We tested these two assumptions in two separate perifusion experiments. Our results clearly indicate that oCRF does not influence the *in vitro* thyroxine (T_4) secretion from the thyroid. The pituitaries of the newly hatched chickens, however, released significant amounts of TSH after *in vitro* treatment with oCRF. Growth hormone and luteinizing hormone secretions were also elevated after the oCRF stimulation period, but this release was less pronounced compared to the TSH release. As a conclusion we postulate that oCRF mediates its effect on the thyroidal status of the chicken through stimulation of TSH release and not through a direct effect on the thyroid.

Keywords : chicken, oCRF, *in vitro*, TSH, T_4.

INTRODUCTION

In vertebrates it is well established that the pituitary produces a thyroid-stimulating hormone (thyrotropin : TSH) which acts directly upon the thyroid gland to stimulate the synthesis and release of thyroxine (T_4) (mammals : Vale *et al.*, 1974 ; birds : Williamson and Davison, 1985 ; Kühn *et al.*, 1988 ; amphibians : Darras and Kühn, 1983 ; reptiles : Lance and Sawin, 1979 ; fish : Milne and Leatherland, 1980). The secretion of this TSH in mammals and birds is regulated by several factors, the best documented being TSH-releasing hormone (TRH) and somatostatin (SRIF), respectively a stimulating and inhibiting hypothalamic factor (Vale *et al.* 1974 ; Drouin *et al.*, 1976 ; Radke and Chiasson, 1977 ; Iqbal *et al.*,

1989). Earlier reports of our research group suggested that ovine corticotropin-releasing factor (oCRF) has also a stimulatory effect on thyroid hormone secretion in the embryonic chicken. T_4 and triiodothyronine (T_3) plasma levels of dwarf and normal chicken embryos were increased after an intravenous (iv) injection of oCRF (MEEUWIS et al., 1989 ; KÜHN et al., 1990). It was however not clear at what level oCRF interacted with thyroid function. It is known that glucocorticoids and adrenocorticotropic hormone (ACTH), whose release in birds is controlled by CRF (ESTIVARIZ et al., 1984 ; CARSIA et al., 1986), depress circulating concentrations of thyroid hormones in respectively posthatch and adult chickens (DECUYPERE et al., 1983 ; WILLIAMSON and DAVISON, 1987 ; MITCHELL et al., 1986). During the last period of the embryonic development, on the contrary, glucocorticoids are reported to increase the T_4 and T_3 plasma concentrations (DECUYPERE et al., 1983). The promptness of the response of circulating thyroid hormone levels after stimulation with oCRF, however, suggests that there may be a direct interaction of oCRF with the thyroidal axis.

The aim of the present study was to investigate if oCRF influences the thyroidal axis through a direct effect on the thyroid gland and/or through the stimulation of TSH release. We tested these two assumptions in two separate series of perifusion experiments : 1) the effect of oCRF on the *in vitro* T_4-releasing activity of the thyroid gland, 2) the response of the pituitary to oCRF. Because of the lack of specific monoclonal antibodies (mAbs) to the chickenTSH (cTSH) β-subunit we used the subtractive strategy published by BERGHMAN et al. (1993) to obtain an indicative cTSH value in the pituitary eluates.

MATERIAL AND METHODS

Animals

One day old male chickens (C1) from a layer strain (Hisex white) were purchased from a commercial dealer (Euribrid, Aarschot, Belgium) and the same day used for the experiments.

Perifusion experiments

In the first experiment the thyroid glands were removed from the chickens and immediately submerged in Gibco 199 medium (M199 : Life Technology, Gent, Belgium). Each pair of thyroids was placed in an individual perifusion chamber, held in a water bath at 37° C, and the perifusion was started with M199 at a constant flow rate of 12 ml per hour. After a preincubation period of 90 min to stabilise the basal secretion of T_4, collection of 1-ml samples (= period of 5 min) from each individual chamber was started. After 30 min of baseline registration, 4 chambers remained perifused with the control medium, the other 8 received M199 containing 10 or 100 nM oCRF (n = 4 per group) (UCB Bioproducts, Braine-l'Alleud, Belgium) for the next 30 min. Afterwards the perifusion continued for another 90 min with the control medium in the absence of any stimulatory agent.

The samples were stored at $-20°$ C prior to the analysis of T_4 by radioimmunoassay (RIA).

During the second experiment 12 pituitaries from newly hatched chickens (C1) were stimulated with 10 or 100 nM oCRF (n = 6 per experimental group). The same protocol was followed (see above), but the post-stimulation period was 120 min instead of 90 min. No baseline control group was added. The samples were kept at $-20°$ C prior to the analysis of cLH, cFSH, cα-subunit and chicken growth hormone (cGH) by RIA.

Radioimmunoassays

T_4 and cGH levels were measured by radioimmunoassay as described before (DARRAS et al., 1991 ; 1992b).

The RIA of chicken α-like immunoreactivity (IR) was carried out as published by BERGHMAN et al. (1993). Briefly, 100 µl of the mAb (1/500,000), validated in binding studies of the mAbs with Reference Tracer Preparation (BERGHMAN et al., 1993), was incubated overnight with 100 µl USDA-cLH-I-1 tracer ($\pm$ 30,000 cpm), 100 µl of the sample or 50 µl of the standard (USDA-cLH-K-3 : 0.78 to 100 ng/ml). In order to obtain equal volumes in the assay, 50 µl M199 was added to the standards. Samples showing concentrations higher than 100 ng/ml were measured again with the same RIA, except in that case 25 µl of the sample and 75 µl M199 were added. The next day we used Sac-Cel anti-mouse (Innogenetics, Zwijndrecht, Belgium) to separate free and bound radioactivity. The total titer of α-containing molecules is than expressed in relative units (ng cLH-like IR/ml).

The homologous RIAs for cFSH and cLH were recently developed and validated (KRISHNAN et al., 1992 ; 1993 ; PROUDMAN, KRISHNAN and BAHR, unpublished results). The radioiodination and the RIA was performed as described by BERGHMAN et al. (1993).

Calculation of an indicative cTSH level in the samples

Specific mAbs to the cTSH-molecule are not available yet because to date researchers have not been successful in isolating this peptide from chicken pituitary. Heterologous polyclonal Abs have been applied to localise TSH-cells in the chicken pituitary (THOMMES et al., 1983). Unfortunately, such Abs tend to have too low affinity with cTSH to allow sensitive measurements by RIA. Therefore we calculated an indicative value for the cTSH levels in our samples using a subtractive strategy published by BERGHMAN et al. (1993). This indicative cTSH level in the samples was obtained by the subtraction of cFSH and cLH levels from the total level of α-like immunoreactive material in the same sample. This indirect method obviously assumes that free α-subunit is not being secreted under physiological conditions (BERGHMAN et al., 1993). Finally, the cTSH concentration in the samples is expressed in relative units because of the lack of homologous standard TSH preparations.

Statistics

For each individual chamber (*e.g.* pair of glands) we calculated (Fig. 1) : 1) basal secretion (BS), 2) the peak value in the response to the stimulator (PV), 3) the stimulation factor (SF), 4) total secretion after stimulation (TS). Statistical analysis between the experimental groups was carried out by one-way-analysis of variance (ANOVA).
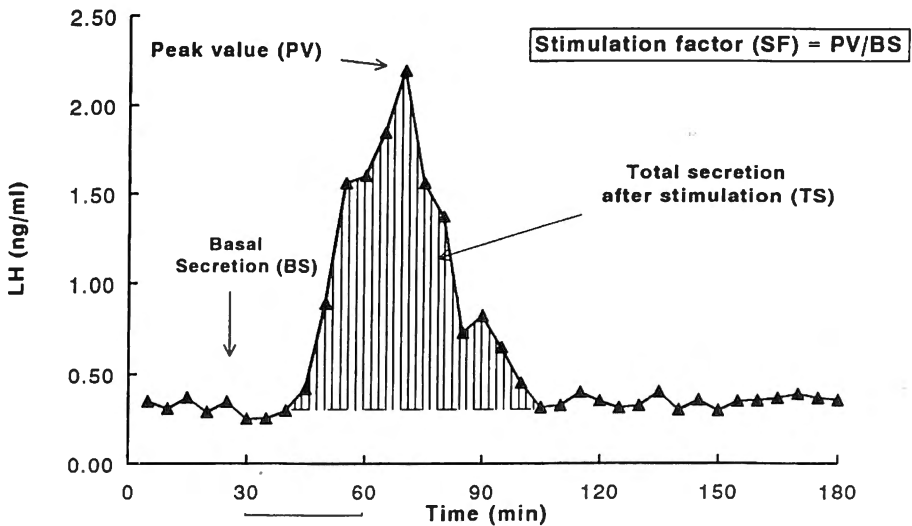

Fig. 1. — Typical individual LH response curve to oCRF in a perifusion experiment. The four different experimental parameters that are calculated, are indicated. The horizontal line beneath the X-axis indicates the stimulation period which is the same as used in the different perifusion experiments.

RESULTS

Experiment 1 : Influence of oCRF on the *in vitro* T_4 release

The *in vitro* T_4 release from thyroids dissected from C1 chickens was not affected by a treatment with 10 or 100 nM oCRF (Fig. 2). The basal T_4 secretion was higher in both oCRF conditions compared to the control group (control : BS = 0.15 ± 0.02 pmol/ml ; 10 nM : BS = 0.25 ± 0.02 pmol/ml ; 100 nM : 0.25 ± 0.02 pmol/ml : $P < 0.05$). However no stimulation of the release was seen in response to an oCRF-treatment as shown by their respective stimulation factors : control : SF = 1.00 ± 0.14 ; 10 nM : SF = 0.92 ± 0.03 ; 100 nM : SF = 1.14 ± 0.03.
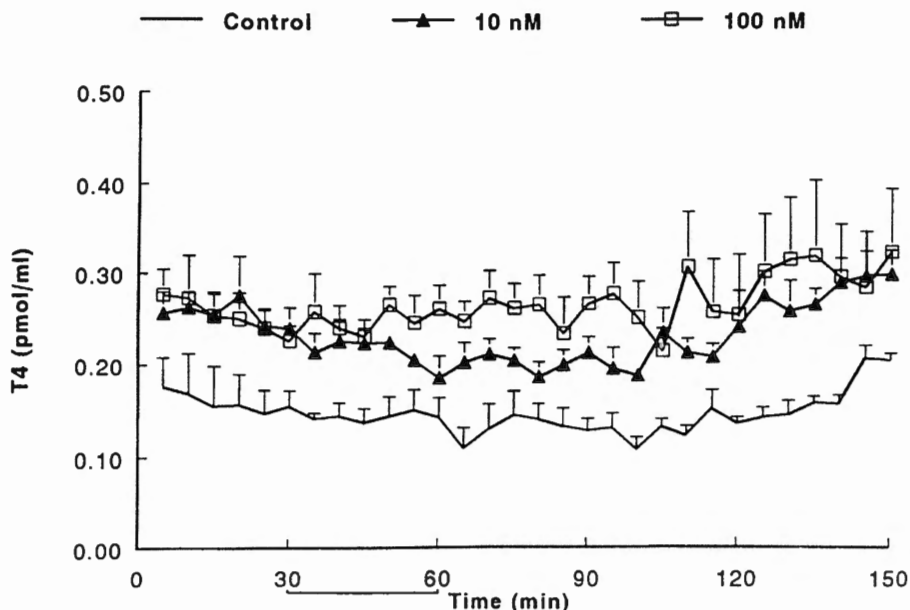
Fig. 2. — *In vitro* T_4 release by thyroid glands of one day old chicks (Cl), stimulated for 30 min with 10 or 100 nM oCRF after a 30 min baseline registration. T_4 levels are measured by RIA. Values shown are mean ± SEM of four individuals. The horizontal line beneath the X-axis indicates the stimulation period.

Experiment 2 : Influence of oCRF on the *in vitro* releasing activity of the chicken pituitary

The effect of oCRF on the α-subunit and LH release is shown in Fig. 3. The oCRF-treatment had a dose-dependent effect on the total amount of α-subunit in the samples. The highest concentration caused a significantly greater SF and TS (Table 1). Also the LH secretion was enhanced, although to a lesser extent compared to α-subunit release. Both doses induced approximately a four-fold increase in LH release. The LH response caused by 100 nM, however, lasted longer, which explains the significantly higher TS in this condition compared to the other experimental group. The amount of cFSH in the samples was either very low or below the detection limit (< 0.20 ng/ml) before, during and after the stimulation period (data not shown). Thus, no stimulatory effect of oCRF on the cFSH-release was noticed. Finally cLH values were subtracted from the α-subunit levels to yield, in each sample individually, an indicative concentration of cTSH. Because of the low or not measurable amount of cFSH we didn't take these data into account for subtraction. As shown in Figure 4 oCRF is a very strong stimulator of the *in vitro* TSH secretion in newly hatched fowl. Due to the wide variation in the individual responses to the highest oCRF concentration, stimulation factors did not differ significantly between the 2 groups, but the total TSH secretion after stimulation showed a clear significant dose-dependent effect of oCRF (Table 2).
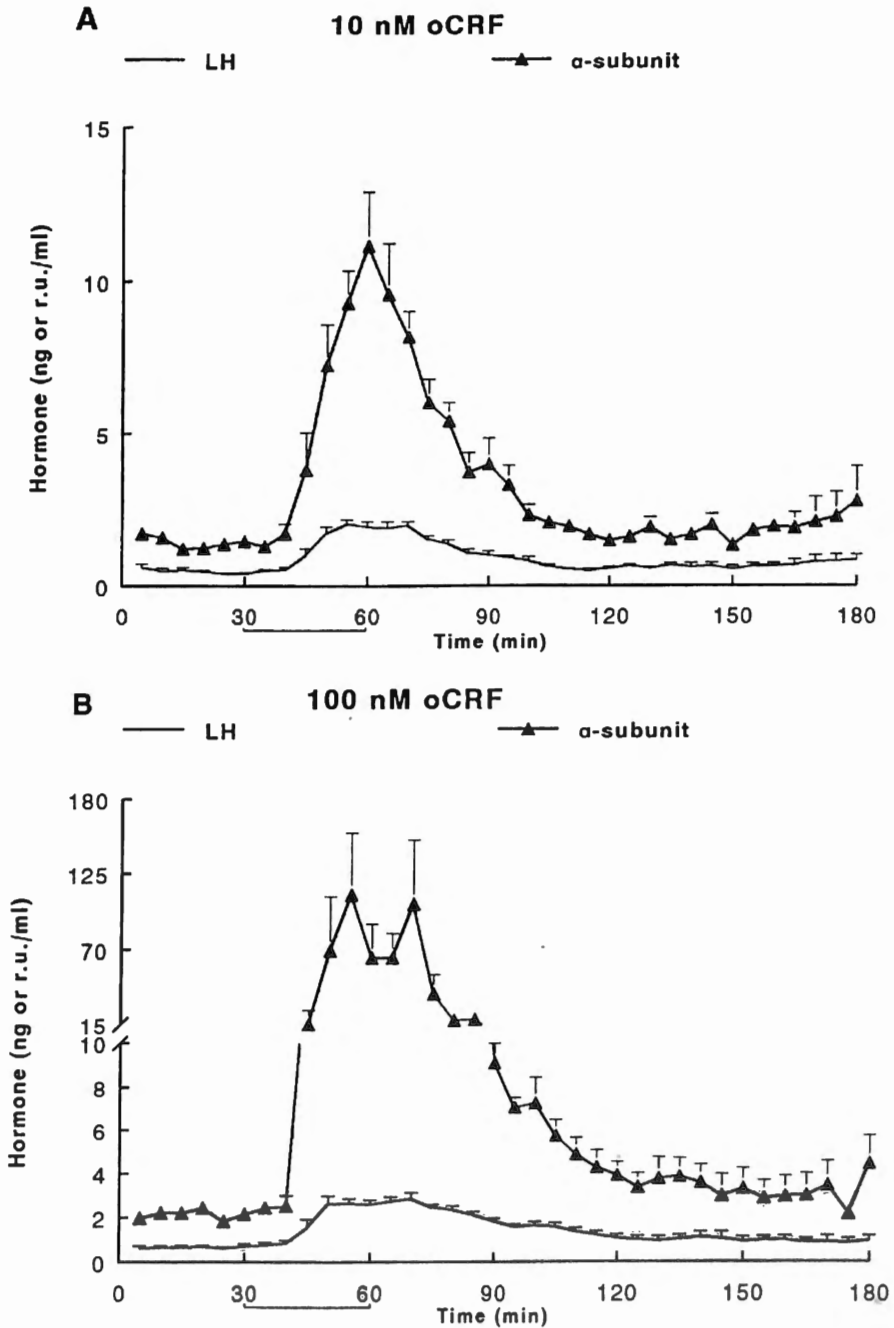
Fig. 3. — *In vitro* LH and α-subunit release by pituitaries of one day old chicks (Cl), stimulated for 30 min with 10 (A) or 100 nM oCRF (B) after a 30 min baseline registration. LH and α-subunit levels are measured by a homologous RIA. Values shown are mean ± SEM of six individuals. The horizontal line beneath the X-axis indicates the stimulation period. The α-subunit levels are expressed in relative units.

TABLE 1

Influence of 10 nM and 100 nM oCRF on the LH (ng/ml) and α-subunit (relative units/ml) secretion in one day old chicks (Cl) (n = 6 per group). Values shown are mean ± SEM. Asterisks indicate differences between the 2 doses (ANOVA : * P < 0.05 ; ** P < 0.01).

	basal secretion (ng or r.u./ml) (BS)	peak value (ng or r.u./ml) (PV)	stimulation factor (SF)	total secretion after stimulation (ng or r.u.) (TS)
LH (ng)				
10 nM oCRF	0.46 ± 0.05	2.01 ± 0.14	4.50 ± 0.21	11.70 ± 0.66
100 nM oCRF	0.69 ± 0.07*	2.86 ± 0.26*	4.23 ± 0.37	18.82 ± 1.57**
α-subunit (r.u.)				
10 nM oCRF	1.30 ± 0.08	11,15 ± 1.73	8.51 ± 1.09	57.08 ± 7.05
100 nM oCRF	2.22 ± 0.23**	110.08 ± 44.94	44.94 ± 16.27*	497.65 ± 131.94**

TABLE 2

Indicative TSH variations in response to 10 nM and 100 nM oCRF in one day old chicks (Cl). Subtractions (αIR-cLH) were calculated for individual animals. Values shown are mean ± SEM of 6 individual subtraction results. Asterisks indicate differences between the 2 doses (ANOVA : **P < 0.01).

	basal secretion (r.u./ml) (BS)	peak value (r.u./ml) (PV)	stimulation factor (SF)	total secretion after stimulation (r.u.) (TS)
10 nM oCRF	0.84 ± 0.04	9.23 ± 1.58	10.85 ± 1.59	45.88 ± 6.56
100 nM oCRF	1.53 ± 0.16**	107.23 ± 44.88	63.63 ± 23.76	478.83 ± 130.58**

The GH secretion (Fig. 5) was hardly influenced by 10 nM oCRF (SF = 1.71 ± 0.17), a concentration of 100 nM oCRF however induced a significantly higher GH release compared to the lower dose (SF = 3.35 ± 0.47 : P < 0.01). The total GH secretion after stimulation differed also significantly in the 100 nM condition (respectively TS = 28.33 ± 6.08 and TS = 116.33 ± 37.68 : P < 0.05).
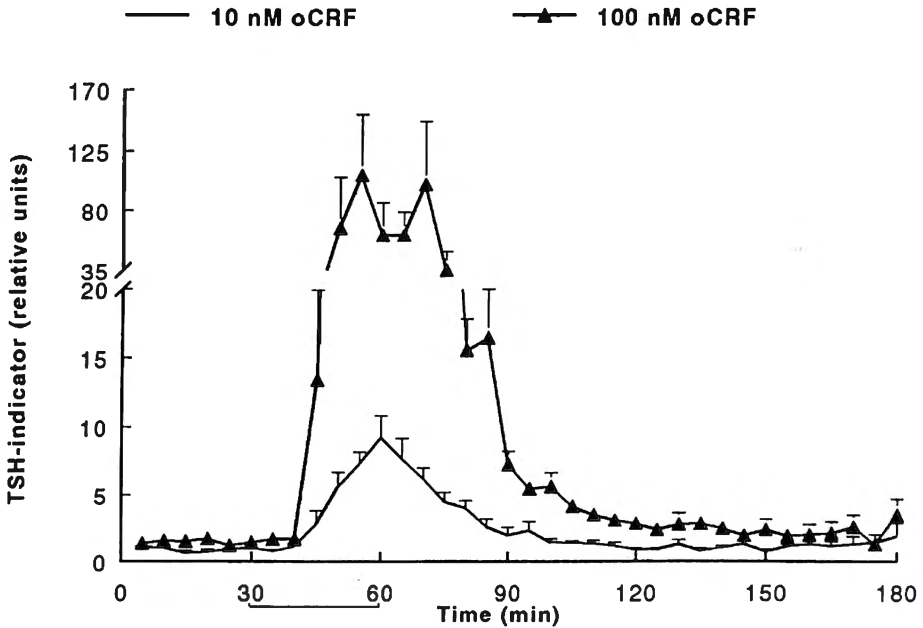
Fig. 4. — Indicative levels of *in vitro* TSH release by pituitaries of one day old chicks (Cl), stimulated for 30 min with 10 or 100 nM oCRF after a 30 min baseline registration. TSH indicator levels are calculated using a subtractive strategy (αIR-cLH). Values shown are mean ± SEM of six individuals. The horizontal line beneath the X-axis indicates the stimulation period.

DISCUSSION

The present study describes the effect of oCRF on the *in vitro* releasing activity of two tissues that are implicated in the thyroidal status of the chicken : the thyroid gland and the pituitary. oCRF is a well-known stimulator of the ACTH-release in mammals, both *in vivo* and *in vitro* (reviewed by RIVIER and PLOTSKY, 1986), and in chickens when added to dispersed adenohypophyseal cells (CARSIA et al., 1986). Due to the lack of an homologous RIA for cACTH, we did not measure the amount of this hormone in our samples. CARSIA et al. (1986) obtained an indicative value for the ACTH concentrations in their experiment using a bioassay. Recently CRF-neurones have been localised in the avian brain (JOSZA et al., 1984 ; KOVÁCS et al., 1989). Also in lower vertebrates oCRF is a potent stimulator of the ACTH release. In fish and anurans this secretagogue enhanced the *in vivo* and *in vitro* ACTH secretion (FRYER et al., 1983 ; CUET et al., 1984 ; TONON et al., 1986). *In vivo* administration of oCRF to a frog species significantly reduced the volume density of the secretory granules in ACTH-cells, taken as an indicator of short-term enhanced hormonal release (MALAGON et al., 1991).
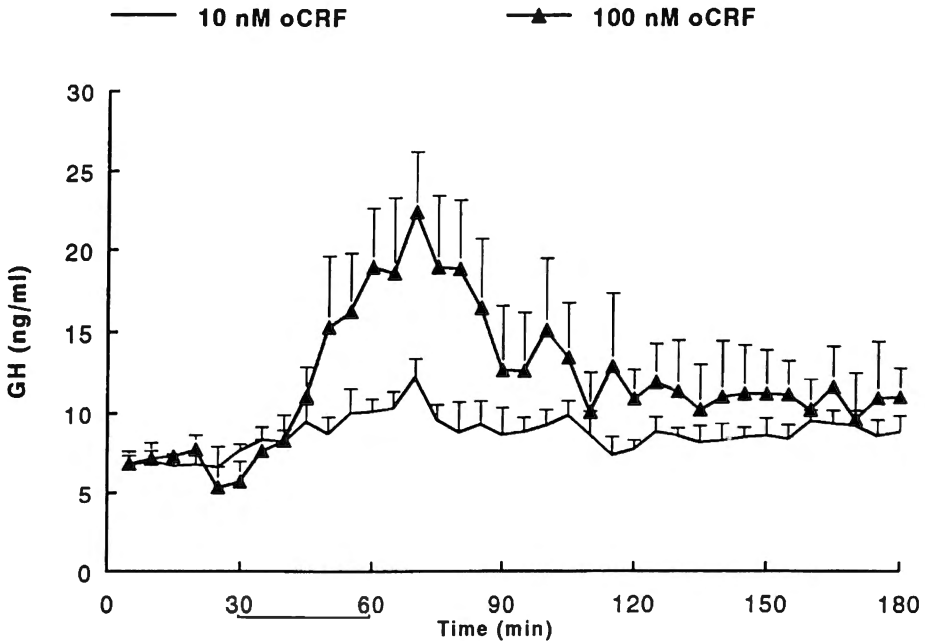
―――― 10 nM oCRF   ―▲― 100 nM oCRF

Fig. 5. — *In vitro* GH release by pituitaries of one day old chicks (Cl), stimulated for 30 min with 10 or 100 nM oCRF after a 30 min baseline registration. GH levels are measured by a homologous RIA. Values shown are mean ± SEM of six individuals. The horizontal line beneath the X-axis indicates the stimulation period.

In mammals oCRF did not change the *in vitro* TSH secretion (VALE et al., 1981). To our knowledge, there are no data available concerning the effect of oCRF on the thyroidal releasing activity in mammals. Our results clearly indicate that oCRF influences the thyroidal axis in the chicken through the stimulation of TSH release as calculated with the subtractive strategy published by BERGHMAN et al. (1993). On the other hand, we did not see a direct effect on the thyroidal T_4 release. Our data correspond with results described in several frog species and in hatchling turtles : each time oCRF induced a rise in the *in vitro* TSH release, without influencing the thyroidal T_4 secretion (DENVER, 1988 ; DENVER and LICHT, 1989a, b ; JACOBS and KÜHN, 1992). MALAGON et al. (1991) observed a decrease in the volume density of the granules of the TSH-cells of a frog species after an oCRF-treatment, indicating an elevation of the TSH release. JACOBS et al. (1988) postulated that LH-releasing hormone (LHRH) also has a TSH-releasing activity in the frog.

oCRF also stimulated the *in vitro* release of other pituitary hormones. The GH secretion was influenced in a dose-dependent manner with the lower dose inducing almost no stimulation. Until this moment GH-releasing factor (GRF) and TRH were considered to be the main GH secretagogues in avian species (HARVEY et al., 1978, 1981 ; LEUNG and TAYLOR, 1983 ; HARVEY et al., 1984). Somatostatin (SRIF) plays an inhibitory role in this process (SCANES and HARVEY, 1989). In young turtles oCRF also stimulated the *in vitro* GH release (DENVER and LICHT, 1989a). RIVIER

and PLOTSKY (1986) mentioned in their review that in mammals oCRF does not have an *in vivo* (iv injections) or *in vitro* GH-releasing activity. In the rat, an intracerebroventricular (icv) oCRF-injection even caused a decrease in the GH plasma concentration (RIVIER and VALE, 1984a). Next to this *in vitro* GH-releasing activity of oCRF in newly hatched fowl, we also observed an increase in the LH release in response to the oCRF-treatment. Gonadotropin-releasing hormone (GnRH) is believed to be the main hypothalamic hormone that releases LH from the pituitary. Our results indicate that in the chicken oCRF is also a candidate for the regulation of the LH secretion. In the rat, authors did not observe any change in the LH plasma levels after an iv oCRF-injection (VALE *et al.*, 1981 ; DONALD *et al.*, 1983 ; RIVIER and VALE, 1984b), while the rhesus monkey responded with a decrease in the LH concentration (GINDOFF *et al.*, 1989). The results after an icv oCRF-injection all indicated a decrease in the LH plasma levels (RIVIER and VALE, 1984b ; PETRAGLIA *et al.*, 1987 ; MAEDA *et al.*, 1994), possibly through its inhibitory effect on the GnRH release (NIKOLORAKIS *et al.*, 1986). We did not find any reports on a LH-releasing activity of oCRF in lower vertebrates. In the turkey there are some indications available that TRH has a LH-releasing activity (WENTHWORTH *et al.*, 1976 ; FEHRER *et al.*, 1985). Although the amounts of cFSH in our samples were very low or not detectable, we can conclude that oCRF does not have an effect on the release of this pituitary hormone. To our knowledge, there are no data available in literature about this specific activity of oCRF in vertebrates.

The *in vivo* relevancy of our results are presently under investigation. The observed *in vitro* TSH-releasing potency of oCRF strengthens the data of MEEUWIS *et al.* (1989) and KÜHN *et al.* (1990), who reported an increase of the circulating thyroid hormone plasma levels after *in vivo* oCRF-treatment in normal and dwarf chicken embryos. Currently, we are focussing on the short-term effects of oCRF on the *in vivo* TSH, GH, LH and circulating thyroid hormones plasma levels in 19-day-old chicken embryos. Since GH can increase T_3 plasma concentrations through a decrease in the T_3 degradation (DARRAS *et al.*, 1992a ; 1993), we also try to estimate the impact of *in vivo* GH release after oCRF on circulating thyroid hormone levels by measuring the activity of deiodinases in several tissues.

In summary, the data presented here suggest that CRF is a potent stimulator of TSH secretion in the domestic fowl while a direct effect of CRF on the thyroid gland can be ruled out. In addition, the study showed for the first time an *in vitro* LH- and GH-releasing activity of CRF in the chicken, although less pronounced then the TSH response.

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