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Effect of Hypothalamic Extract on the Prolactin Release from the Bullfrog Pituitary Gland with Special Reference to Thyrotropin-Releasing Hormone (TRH)

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ABSTRACT—Acid extract of bullfrog hypothalami but not of rat hypothalami stimulated the release of prolactin (PRL) from the bullfrog pituitary gland *in vitro*. Since frog hypothalamus is known to contain thyrotropin-releasing hormone (TRH), a stimulator of PRL release, much more than rat hypothalamus, experiments were performed to determine whether PRL-releasing activity of the frog hypothalamic extract is derived from TRH it contains. PRL-releasing activity in the hypothalamic extract was nullified by incubation with bullfrog plasma. When the extract was fractionated on Sephadex G-25 chromatography, significant PRL-releasing activity was found in the fraction which is presumed to contain TRH. The activity of the hypothalamic extract was markedly reduced by co-incubation with IgG fraction separated from antiserum to TRH. These results indicate that TRH is one of the major PRL-releasing factors in the hypothalamic extract.

INTRODUCTION

Acid extract of the amphibian hypothalamus is known to stimulate the release of prolactin (PRL) from amphibian pituitary glands [1–3]. However, little is known about the identity of PRL-releasing factor in the hypothalamic extract. On the other hand, synthetic thyrotropin-releasing hormone (TRH) shows a potent PRL-releasing activity in amphibians [2, 4–7] as well as in mammals [8]. In amphibians, it is not clear whether TRH regulates TSH secretion. According to the results obtained by most of the investigators, there is no indication that synthetic TRH stimulates TSH release from amphibian pituitary gland [9–12], while some recent data pointed out the possibility that TRH induces TSH release in the amphibian [13]. It has been reported that hypothalamic TRH concentration is much higher in amphibians than in mammals [11, 14–16]. Immunohistochemical study of the bullfrog hypothalamus revealed that TRH neurons exist in the hypothalamus and their axons terminate in the median eminence, suggesting that TRH neurons have a physiological role in the regulation of adenohypophyseal hormone release [16].

The present investigation was undertaken to ascertain whether the PRL-releasing activity in the hypothalamic extract is due to TRH. A preliminary report has been published elsewhere [3].

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MATERIALS AND METHODS

Incubation of pituitary gland

Adult bullfrog (*Rana catesbeiana*) weighing 250–400 g were sacrificed by decapitation. The anterior pituitary gland was removed, weighed and hemisected. Two hemipituitaries were placed in a glass vial containing 200 μl of 67% Eagle MEM (Nissui Seiyaku Co., Ltd., pH 7.4). The vials were incubated in a Dubnoff metabolic incubator in an atmosphere of 95% O_2–5% CO_2. After 1 hr of preincubaion, the medium was replaced with fresh one containing a test substance. Incubation was

carried out for 8 hr at 25°C, since it was previously verified that bullfrog pituitaries incubated in the same condition as described above continue to release PRL until at least 28 hr at a linear rate [7].

Radioimmunoassay

PRL in the medium was measured by a homologous radioimmunoassay for bullfrog PRL developed in Yamamoto and Kikuyama [17].

Preparation of tissue extract

Fresh hypothalami were homogenized in cold 0.1 N HCl (50 μl/hypothalamus) with a teflon homogenizer and centrifuged at 10,000×*g* for 30 min. The supernatant was neutralized with 1 N NaOH and recentrifuged. Acid extracts of frog cerebrum, frog neuro-intermediate lobe and rat hypothalamus were prepared in the same manner.

Inactivation of PRL-releasing activity of hypothalamic extract by frog plasma

Fifty microliters of the extract (one hypothalamic equivalent) was mixed with 50 μl of bullfrog plasma or distilled water and incubated for 3 hr at 37°C. After incubation, PRL releasing activity of this mixture was tested.

Chromatography

Hypothalamic extract, derived from 200 hypothalamic flagments was filtered through Millipore filter (HAWP) and applied to a 1.5×110 cm Sephadex G–25 column. Each fraction consisting of 3 ml was collected by eluting with 0.1 N acetic acid. ^{3}H-TRH (1-proline-2, 3-H(N)-TRH, New England Nuclear) was also chromatographed to determine the position in which TRH is eluted. Fractions were assembled into three pools; fraction I (FI) eluted faster than TRH, fraction II (FII) eluted with TRH and fraction III (FIII) eluted later than TRH. The pooled fractions were lyophilized, dissolved in the incubation medium and tested for PRL-releasing activity.

Inactivation of PRL-releasing substance by IgG from anti-TRH serum

Immunoneutralization of hypothalamic TRH was performed by the use of IgG fraction from anti-TRH serum which had been prepared by immunizing rabbit with TRH conjugated to bovine serum albumin according to the procedures described previously [18]. Protein A (Pharmacia Fine Chemicals) was combined with CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) by the method of Miller and Stone [19]. Anti-TRH serum or normal rabbit serum was applied to 0.8×4.5 cm protein A–Sepharose column. The IgG fraction was dialized, lyophilized and dissolved in 50 mM Hepes buffer containing 0.7% NaCl (HBS). Hypothalamic extract was incubated with the solution of IgG from anti-TRH serum, from normal rabbit serum (NRS) or HBS at 4°C for 48 hr. After incubation, the medium was centrifuged. To test the PRL-releasing activity, aliquot of the supernatant was added to the medium in which pituitaries were placed. One milliliter of medium used for the test contained the extract from 0.2 hypothalamus which had been incubated with IgG obtained from 60 μl of antiserum or NRS. It has been ascertained that 1 μl of the antiserum can inactivate nanogram quantities of TRH.

RESULTS

The response of bullfrog pituitaries to various doses of frog hypothalamic extract is shown in Figure 1. Hypothalamic extract, equivalent to 0.01–1 hypothalamic fragment stimulated the release of PRL from bullfrog pituitary gland in a dose-dependent manner. The dose of 0.1 and 1 hypothalamic equivalent per 1 ml medium caused a significant increase in the PRL release.

Effect of rat hypothalamic extract on the PRL release from the bullfrog pituitary gland was examined. No significant difference in the amount of PRL released into the medium was observed between the control medium (300±39 ng/mg pituitary) and the medium containing extract from one rat hypothalamus per milliliter (349±53 ng/mg pituitary).

PRL-releasing activities of extracts from the hypothalamus, cerebrum and neuro-intermediate lobe of the pituitary gland were shown in Figure 2. Addition of the extract from the hypothalamus or neuro-intermediate lobe to the medium significantly enhanced the release of PRL. The extract from

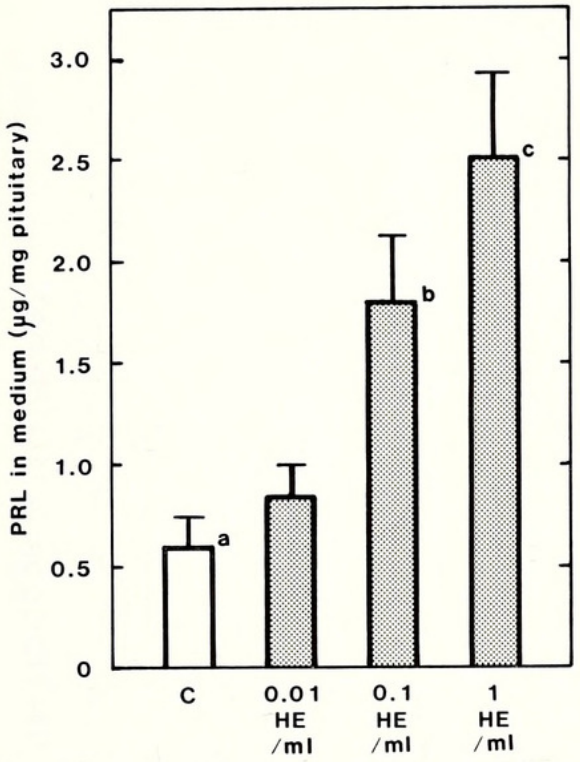


FIG. 1. Effect of various doses of hypothalamic extract (HE) on PRL release. One milliliter of medium contains acid extract from 0.01–1 hypothalamus. C, control medium. Each value represents mean ± SEM for 7 determinations. Significance of difference (analysis of variance): a vs b, a vs c, $P<0.001$.

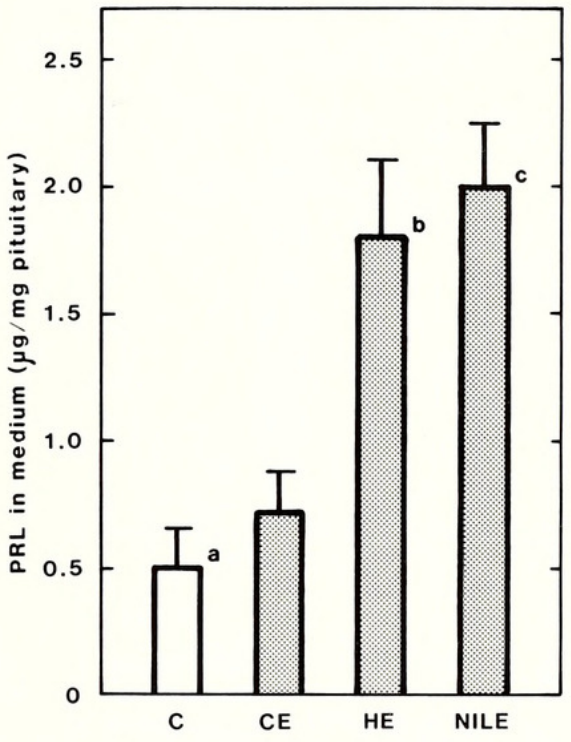
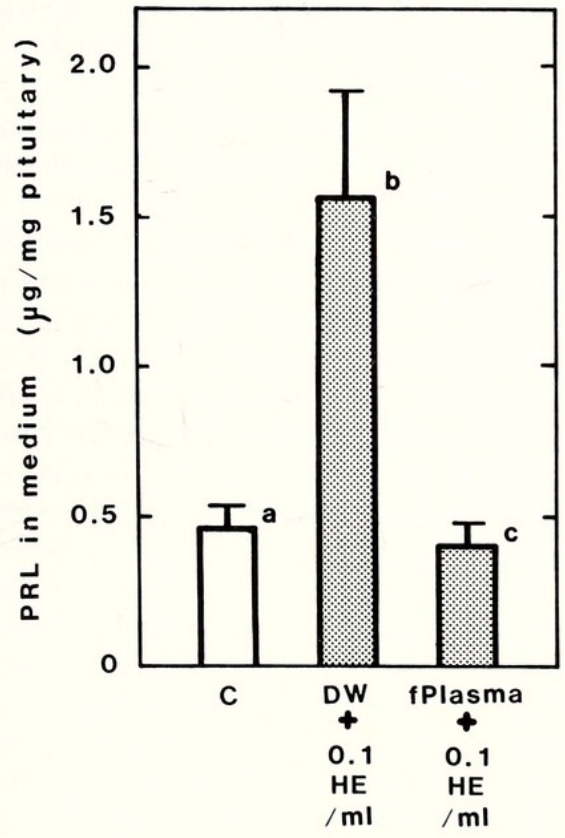


FIG. 2. Effect of extract of cerebrum (CE), hypothalamus (HE) and neuro-intermediate lobe (NILE) on PRL release. One milliliter of medium contains extract from 3 mg of each tissue. Each value represents mean ± SEM for 7 determinations. Significance of difference (analysis of variance): a vs b, a vs c, $P<0.001$.



the cerebrum caused a slight increase, but this increase was statistically not significant.

When the hypothalamic extract was incubated with bullfrog plasma, the PRL-releasing activity was completely lost (Fig. 3).

Figure 4 shows the profiles of Sephadex G–25 chromatography of the hypothalamic extract and the distribution of radioactivity of ^{3}H-TRH when chromatographed. Among the three pooled samples, FII exhibited a marked stimulatory effect on the PRL release, while FIII had no releasing activity and FI had a slight but not significant

FIG. 3. Effect of bullfrog plasma on PRL-releasing activity of bullfrog hypothalamic extract. One milliliter of medium contains extract from 0.1 hypothalamus subsequently incubated with distilled water (DW) or bullfrog plasma as described in Materials and Methods. Each value represents mean ± SEM for 7 determinations. C, control medium. Significance of difference (analysis of variance): a vs b, b vs c, $P<0.001$.

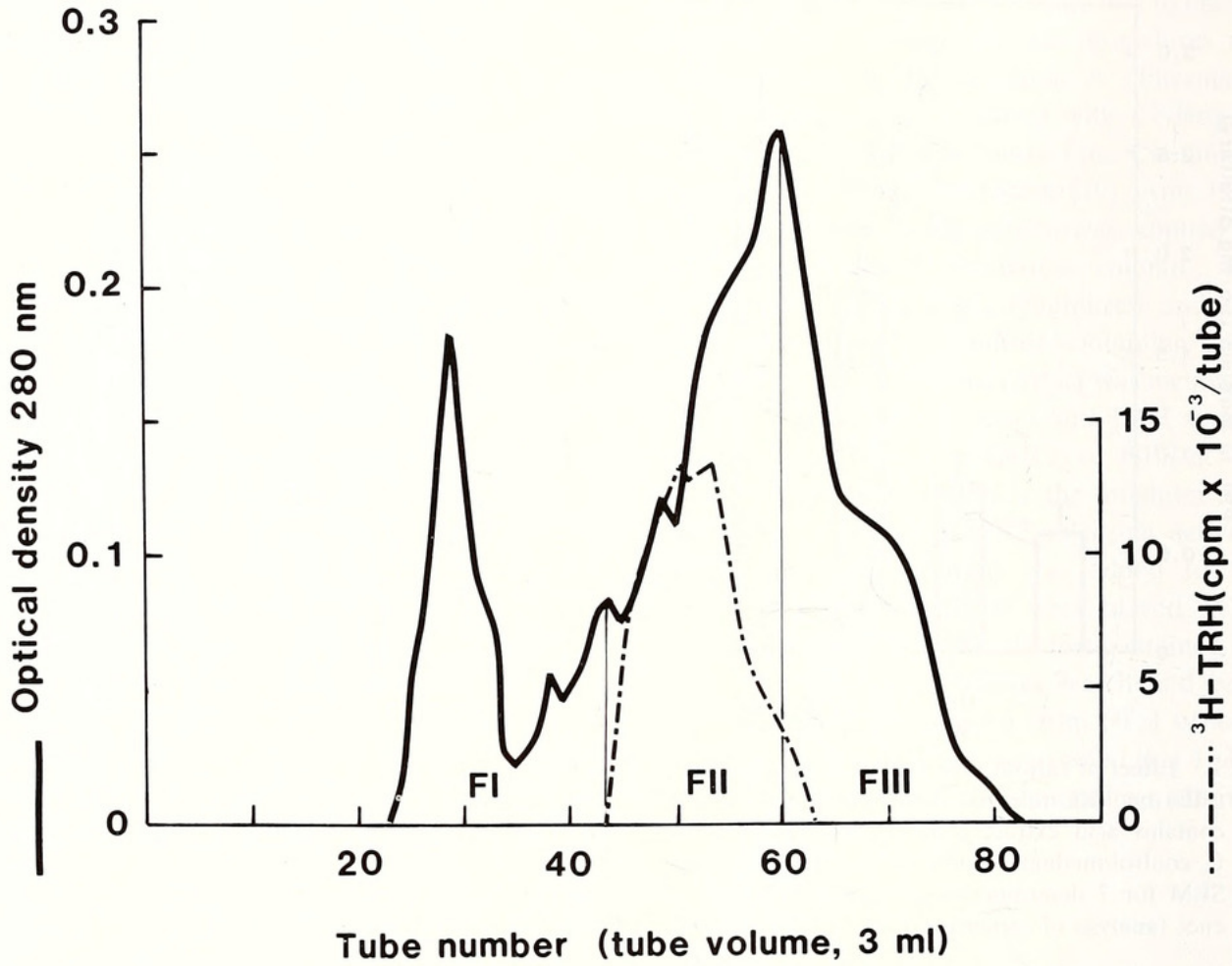


FIG. 4. Sephadex G–25 column (1.5×110 cm) chromatography of bullfrog hypothalamic extract and of ^{3}H-TRH. Absorbance at 280 nm is denoted by the continuous line and radioactivity of ^{3}H-TRH by the broken line.

releasing activity (Fig. 5).

Figure 6 shows the effect of immunoneutralization of TRH in the hypothalamic extract on the PRL release. Hypothalamic extract incubated with either IgG from NRS or HBS stimulated the PRL release. Incubation of the hypothalamic extract with IgG from anti-TRH serum considerably diminished the PRL-releasing activity of the hypothalamic extract.

DISCUSSION

It was demonstrated by the present experiment that the frog hypothalamic extract is effective in promoting PRL release from the bullfrog pituitary gland *in vitro*, while the rat hypothalamic extract is ineffective. It has also been reported that the frog hypothalamic extract stimulates the release of PRL from the rat pituitary gland [20]. These results indicate that the effect of frog hypothalamic extract on the PRL release is predominantly stimulatory.

TRH is known to have a potent PRL-releasing activity in amphibians [2–6] as well as in mammals [8]. Among the three fractions separated from the bullfrog hypothalamic extract by Sephadex G–25 chromatography, the fraction (FII) which is presumed to contain TRH had the strongest PRL-releasing activity. In the present experiment, no attempt was made to eliminate PRL-inhibiting factors presumed to be present in the hypothalamic extract. Dopamine existing in the frog hypothalamus has been postulated to be a PRL-inhibiting factor [5, 21–25]. According to our test-run on Sephadex G–25 column, the monoamine was eluted later than TRH, indicating that dopamine will be included in FIII. Immunoneutralization of the hypothalamic extract

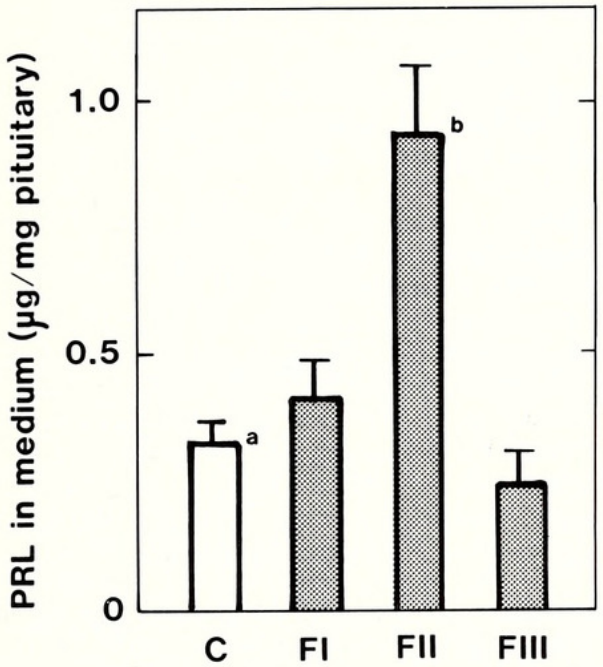


FIG. 5. Distribution of PRL-releasing activity among fractions obtained by gel-filtration of bullfrog hypothalamic extract on Sephadex G–25. One milliliter of medium contains each fraction derived from 0.1 hypothalamus. Each value represents mean ± SEM for 7 determinations. Significance of difference (analysis of variance): a vs b, $P<0.001$.

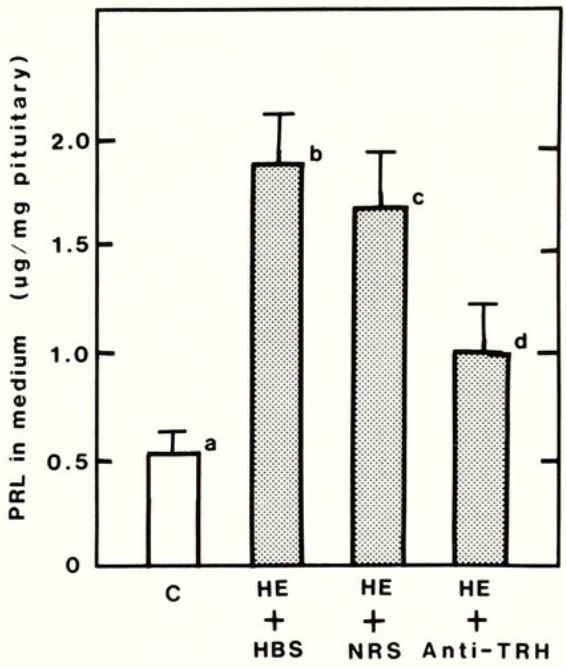


FIG. 6. Effect of immunoneutralization of hypothalamic TRH on PRL release. One milliliter of medium contains acid extract from 0.2 hypothalamus which had been subsequently incubated with 60 μl of 50 mM Hepes buffer containing 0.7% NaCl (HBS), IgG from normal rabbit serum (NRS) or IgG from anti-TRH serum. Each value represents mean ± SEM for 7 determinations. C, control medium. Significance of difference (analysis of variance): a vs b, a vs c, b vs d, c vs d, $P<0.001$; a vs d, $P<0.01$.

with IgG separated from anti-TRH serum resulted in a considerable degradation of PRL-releasing activity. These results strongly suggest that PRL-releasing activity in the hypothalamic extract is largely derived from TRH it contains. In perinatal rats, it has also been demonstrated that the PRL-releasing activity in the hypothalamic extract is attributable to the existing TRH [26].

The PRL-releasing activity of the hypothalamic extract was completely abolished by incubation with frog plasma. Preliminary study also revealed that NRS as well as anti-TRH serum inactivated the PRL-releasing activity of the hypothalamic extract. This may be the result of enzymatic degradation of substances bearing PRL-releasing activity. It is well known that TRH is degraded rapidly in the blood [27]. Accordingly, we used IgG fraction instead of antiserum for immunoneutralization.

The present experiment revealed that extract of neuro-intermediate lobe tissue possesses a marked PRL-releasing activity. Neuro-intermediate lobe tissue as well as the hypothalamus of bullfrogs contains a considerable amount of TRH [15, 16]. Accordingly, the stimulatory effect of the extract from neuro-intermediate lobe may partly be due to TRH. In mammals, it has been reported that extract of posterior pituitary or hypophyseal stalk contains a PRL-releasing activity and that the substance in the extract is distinct from TRH [28–31].

In the present experiment, an excess amount of IgG from anti-TRH serum was applied for the immunoneutralization of TRH in the hypothalamic extract, taking into consideration of the TRH content in the bullfrog hypothalamus [16]. However, complete depression of PRL-releasing activity was not observed. This suggests the existence of PRL-releasing factors other than TRH in the frog hypothalamic extract. Several substances such as vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI) are known to stimulate PRL release from the pituitary gland

in amphibians [32] as well as in mammals [33–36]. Identification of other PRL-releasing substances in the bullfrog hypothalamus is under way.

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