Enzymatic Synthesis of Prolactin-Releasing Factor (PRF) by Rat Hypothalamic Incubates and by Extracts of Rat Hypothalamic Tissue: Evidence for "PRF Synthetase"[1] (37334)

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Nonribosomal enzymatic synthesis of thyrotropin releasing hormone (TRH) by porcine and rat hypothalamic extracts has previously been demonstrated in this laboratory by means of labeled amino acid incorporation studies (1–4) and by the formation *de novo* of biologically active TRH by hypothalamic extracts (3, 4). These observations led us to consider the possibility that one or more of the other hypothalamic hypophysiotropic hormones might also be formed by enzymatic systems analagous to those responsible for TRH biosynthesis, and that the biosynthesizing activity might be demonstrable (as in the case of TRH) by the formation of releasing hormone detectable by bioassay. Studies were, therefore, carried out which demonstrated the formation of bioassayable luteinizing hormone releasing hormone (LRH) (4, 5), and of growth hormone-releasing hormone (GH-RH) (6) by rat hypothalamic fragments and by soluble hypothalamic extracts in the presence of appropriate amino acids and cofactors.

In this paper, we report the extension of our work on releasing factor biosynthesis to the study of prolactin releasing factor (PRF). The existence of PRF has not been accepted as generally as has the existence of the other postulated releasing factors (Nicoll *et al.* (7) and Valverde-R (8)). An assay for PRF has recently been developed in this laboratory (9), utilizing as a measure of activity the increment in plasma radioimmunoassayable prolactin level in estrogen–progesterone treated male rats after intravenous injection of porcine and rat hypothalamic extracts. This bioassay has been used to study formation of PRF by incubates of rat hypothalamic fragments and by extracts of rat hypothalamic tissue.

Methods. Whole tissue incubation. Hypothalamic tissue from male Sprague–Dawley rats (Blue Spruce Farms), 250–350 g body wt was removed in three fragments: stalk median eminence, ventral and dorsal hypothalamus. The fragments removed from animals in pairs were incubated together in 1.0 ml of Krebs–Ringer bicarbonate buffer, pH 7.2, in a shaking incubator under an atmosphere of 95% oxygen and 5% carbon dioxide as previously described (1, 3). At the end of the incubation, the tissues were homogenized in the media, the media were pooled, centrifuged at 3000 rpm for 20 min and the supernatant bioassayed in a dosage equivalent to five hypothalami per assay animal. In another incubation, carried out simultaneously, Puromycin was added to the incubation media in a concentration of 200 μg/ml, a concentration chosen because it produces almost complete inhibition of new protein formation (3). For controls, hypothalamic fragments were similarly dissected and kept frozen in buffer in concentrations corresponding to the fragments which had been incubated.

Extract incubation. Freeze-dried rat hypothalamic tissue (National Pituitary Distribution Agency, National Institutes of Health), weighing 4.65 mg per rat hypothalamic

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fragment, was homogenized in 0.01 *M* phosphate buffer, pH 7.4, in a Virtis homogenizer. The amount of tissue and buffer was chosen to give a final concentration of 10 or 20 rat hypothalami/ml. Crude extract after homogenization was centrifuged at 3000 rpm for 30 min. In addition, in Expts. 3 and 4 (Table III), the supernatant was further centrifuged for 20 hr at 100,000*g* to sediment particles including ribosomes. Extracts were dialyzed overnight against 0.01 *M* phosphate buffer (pH 7.4) in the cold to free them from preformed storage PRF and from dialyzable cofactors. In each experiment part of the extract was incubated in the presence of a mixture of 20 amino acids, each in a final concentration of 10^{-3} *M*, and ATP and $MgSO_4$, each of the latter in a final concentration of 10^{-4} *M*. The following amino acids were added: isoleucine, lysine, phenylalanine, pyroglutamic acid, cysteine, cystine, proline, methionine, aspartic acid, arginine, glutamine, histidine, glycine, valine, leucine, tyrosine, tryptophan, serine, alanine, and threonine. Control incubations contained the hypothalamic tissue but the amino acids and cofactors were replaced by buffer. In Expts 3 and 4 (Table III), the requirements for ATP and amino acids were also tested separately. Incubations were carried out with shaking at 37°C for one hour under a controlled atmosphere of 95% O_2–5% CO_2, and the synthesis stopped by the addition of equal volumes of absolute methanol. The samples were mixed well, centrifuged at 3000 rpm for 30 min and the supernatants evaporated to dryness under an air stream. The residual material was taken up in saline for intravenous injection into assay animals. In the first and second experiments using hypothalamic extracts (Table II), a total of 60 rat hypothalami in 6 ml of phosphate buffer was used for each incubation group (treated or untreated). The incubation mixture was then extracted and taken up in saline as described above to provide the equivalent of 10 hypothalami per assay animal in an injection volume of 0.5 ml each. In the third and fourth experiments, particulate-free extracts were used and prepared by prolonged centrifugation. In the third experiment, a total of 40 hypothalami were incubated in 4 ml for each incubation group; an equivalent of 10 rat hypothalami were injected into each assay rat. In the fourth experiment, a total of 80 rat hypothalami were incubated in 4 ml for each incubation group. The equivalent of 20 rat hypothalami were injected into each assay rat.

Bioassay of PRF. The bioassay for PRF depends upon the change in plasma radioimmunoassayable prolactin at 10 min after intravenous injection of test substance into male Sprague–Dawley rats, 250–350 g body wt pretreated for three days with estradiol benzoate, 50 μg, and progesterone, 25 mg daily (9). Assays were carried out under sodium pentobarbital anesthesia (50 mg/kg body wt, ip); in two experiments (Expts 2 and 4) the animals were also given reserpine, 0.5 mg/kg body wt, ip 24 hr prior to assay. Heparinized blood samples were removed from the external jugular vein immediately before and 10 min after intravenous injection of hypothalamic extract. Plasma was separated and kept frozen until immunoassay using antibody to rat prolactin and rat prolactin standards provided by the National Pituitary Agency, and a preparation of rat prolactin given us by Dr. Stanley Ellis was used as iodinated antigen.

Results. Biosynthesis of prolactin releasing factor by rat hypothalamic fragments incubated in vitro *(Table I).* PRF activity of 5 rat hypothalamic fragments frozen immediately after removal could not be detected, but a similar amount of hypothalamic tissue incubated in buffer did induce the release of prolactin. The addition of puromycin to the incubation flask in a concentration of 200 μg/ml did not prevent the formation of bioassayable PRF.

Biosynthesis of PRF by rat hypothalamic extract in the presence of amino acids and cofactors. Dialyzed hypothalamic extracts incubated alone in the absence of amino acids and cofactors for one hour had no detectable PRF activity (Tables II, III). On the other hand, in the presence of amino acids, ATP and $MgSO_4$ hypothalamic extracts form significant amounts of bioassayable PRF. Formation of PRF activity can be demonstrated even when

TABLE I. Biosynthesis of Prolactin Releasing Factor by Rat Hypothalamic Fragments Incubated *in Vitro* and Assayed in Estrogen-Progesterone Treated Male Rats.

	No. of assay animals	Increment in plasma prolactin in assay animals at 10 min (ng/ml ± SE)	Significance of difference between incubated and frozen control (p value)
Frozen hypothalami	4	−2.2 ± 1.4	
Incubated hypothalami	4	27.0 ± 12.9	<0.05
Incubated hypothalami + Puromycin 200 μg/ml	4	57.3 ± 34.1	<0.05

the extracts have been freed of particulates by prolonged high-speed centrifugation (Table III). In experiments using particulate-free extracts it has been further shown that omission of either amino acids or of ATP separately (in the presence of hypothalamic extract and Mg^{2+}) was sufficient to prevent new PRF formation (Table III).

Discussion. The experiments reported here demonstrate that incubates of rat hypothalamic tissue and rat hypothalamic extracts synthesize PRF. The specificity of the PRF assay must be commented on. We have previously found that vasopressin induces prolactin release in the estrogen–progesterone treated male rat (9), but biosynthesis of this octapeptide can be excluded as a possible cause of the PRF effects observed on the basis of the work of Sachs *et al.* (10), who have demonstrated that vasopressin is formed on ribosomes. Although TRH is a potent releaser of prolactin in the human being (11, 12), and in rat pituitary tumor cultures (13), the effects observed in this experiment cannot be attributed to TRH biosynthesis because of our demonstration that TRH is ineffective as a PRF in the bioassay system used (9). The ineffectiveness of TRH as a prolactin releaser in the rat has been shown also by Lu *et al.* (14).

Synthesis by hypothalamic incubates is not blocked by a concentration of puromycin shown (3) to be sufficient to produce almost complete blockade of new protein synthesis. This finding suggests that the appearance of new PRF in this system is probably not the result of ribosomal protein synthesis. Further evidence that PRF synthesis is not mediated by ribosomes is the demonstration that soluble, particulate-free extracts are also capable of bringing about the same synthetic reaction. Since both ATP and amino acids are required for the formation of PRH activity, it appears reasonable to conclude that PRF is a polypeptide, the formation of which is ATP dependent. It is proposed therefore that

TABLE II. Enzymatic Synthesis of Prolactin Releasing Factor by Rat Hypothalamic Extract in the Presence of Amino Acids and Cofactors.

	No. of assay animals	Increment in plasma prolactin in assay animals at 10 min (ng/ml ± SE)	Significance of difference between control and extract plus additions (p value)
Experiment 1			
Extract control	6	−4.4 ± 4.8	
Extract + amino acids + ATP + $MgSO_4$	6	9.5 ± 3.2	<0.01
Experiment 2			
Extract control	6	6.0 ± 3.7	
Extract + amino acids + ATP + $MgSO_4$	6	25.1 ± 6.0	<0.001

TABLE III. Synthesis of Prolactin Releasing Factor by a Ribosome-Free Extract of Rat Hypothalamic Tissue in the Presence of Amino Acids and Cofactors.

	No. of assay animals	Increment in plasma prolactin in assay animals at 10 min (ng/ml ± SE)	Significance of difference between control and extract plus additions (p value)
Experiment 3			
Extract control	4	−8.32 ± 4.59	
Extract + amino acids + ATP	4	13.88 ± 4.90	<0.01
Extract + amino acids — ATP	4	0.15 ± 3.44	NS
Extract — amino acids + ATP	4	−3.40 ± 3.61	NS
Experiment 4			
Extract control	4	6.8 ± 12.0	
Extract + amino acids + ATP	4	68.3 ± 4.8	<0.001
Extract + amino acids — ATP	4	24.3 ± 5.5	NS
Extract — amino acids + ATP	4	9.0 ± 9.7	NS

the PRF synthesizing system of hypothalamic tissue be termed "PRF synthetase." The system is probably analogous to the TRH synthetase system (1–5) and LRH synthetase (4, 5) systems previously reported. GH-RH activity has also been shown to be formed in a similar manner (6). The formation of PRF by hypothalamic tissue gives further support to the view that this substance is a physiologically important component of the prolactin regulatory system.

Summary. Prolactin-releasing factor (PRF), activity of rat hypothalamic fragments was increased after incubation in Krebs–Ringer bicarbonate buffer, an effect observed even when puromycin in a concentration sufficient to block new protein synthesis was added. In the presence of a mixture of twenty naturally occurring amino acids, ATP and MG^{2+}, a dialyzed ribosome-free extract of rat hypothalamic tissue showed an increased amount of PRF activity as compared with extracts incubated in the absence of cofactors and amino acids. These observations suggest that PRF is formed by a mechanism not involving new protein synthesis. Since PRF is formed by soluble hypothalamic extracts in the presence of ATP and amino acids, it is proposed that this hypothalamic hypophysiotropic hormone is a polypeptide and is biosynthesized by a "PRF Synthetase."

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