EVIDENCE FOR PEPTIDE NATURE OF LH AND FSH-RELEASING HORMONES*

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Summary. The effects of several chemical and enzymatic treatments on the biological activity of highly purified porcine luteinizing hormone-releasing hormone (LH-RH) and follicle-stimulating hormone-releasing hormone (FSH-RH) (1) were studied in an attempt to define the chemical nature of these hypothalamic hormones. The results obtained are consistent with the concept that LH-RH/FSH-RH activity is due to small basic polypeptide(s), but a conclusive differentiation between LH-RH and FSH-RH cannot yet be made.

The work of various investigators clearly established the presence in hypothalamic extracts of rats, sheep, pigs, cattle and humans of luteinizing hormone-releasing hormone (LH-RH) and follicle-stimulating hormone-releasing hormone (FSH-RH) capable of stimulating the release of LH and FSH from the pituitary (1-7). However the chemical properties of LH-RH and FSH-RH are poorly defined and there is much disagreement as to their chemical nature and their stability to chemical and enzymatic treatments (8). Moreover, another problem arose recently when porcine LH-RH obtained in a high state of purity was shown to stimulate in rats the release not only of LH, but also of FSH (6, 9). In addition porcine LH-RH stimulated the release of both LH and FSH in humans (10). It is not clear whether this FSH-RH activity is intrinsic to

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porcine LH-RH or whether it represents a contamination with FSH-RH. One of us consequently suggested that one hypothalamic hormone is responsible for the release of both FSH and LH from the pituitary (6, 11). The experiments described here were designed to shed more light on the properties of porcine LH-RH and FSH-RH and, if possible, to obtain a differentiation in their behavior to proteolytic enzymes and various chemical treatments.

MATERIALS AND METHODS

LH-RH/FSH-RH was prepared from 165,000 fragments of pig hypothalami. For the purification, the following steps were utilized: gel filtration on Sephadex G-25 columns, extraction of the LH-RH active fractions with phenol, followed by chromatography and rechromatography on carboxymethylcellulose (CMC) columns. This was followed by free flow-electrophoresis at pH 6.3, countercurrent distribution in a system of 0.1% acetic acid: 1-butanol:pyridine = 11:5:3 (v/v) and partition chromatography (1, 6).

The digestions with trypsin, chymotrypsin, subtilisin, carboxypeptidase A and B, leucine aminopeptidase and aminopeptidase M were all carried out in 0.1 M ammonium acetate buffer, pH 8.1. The incubation with pepsin was performed in 0.01 M hydrochloric acid. The digestion with papain was carried out in 0.1 M ammonium acetate buffer at pH 5.0, in an atmosphere of H_2S and in the presence of 0.05 mM EDTA. The reactions with diazotized sulfanilic acid and with N-bromosuccinimide were performed as suggested by Bailey (12) and Shaltiel and Patchornik (13) respectively. For the treatment of LH-RH with nitrous acid, 25 µl 0.4% aqueous sodium nitrite were mixed with 2.5 µg LH-RH in 25 µl 1 N acetic acid and allowed to stand for 2 hrs. The reaction with ninhydrin was carried out by adding 10 µl 2% ninhydrin in a mixture of ethanol: 4 M sodium acetate buffer pH 5.0 (1:3) to 2.5 µg LH-RH and incubating over-

night at 37°. The reaction with 2-hydroxy-5-nitrobenzyl bromide was performed as recommended by Koshland et al. (14). Partial acid hydrolysis was carried out in 0.9 M HCl for 1 hour at 100°. In all cases, control reactions were performed by omitting LH-RH.

LH-RH activity was determined in vivo by stimulation of release of LH in ovariectomized rats pretreated with estrogen and progesterone (15). LH concentration in the serum of assay rats was determined by the radioimmunoassay described by Niswender et al. (16). The increase of serum LH levels after injection of samples was used as the index of LH-RH activity. FSH-RH activity was measured by a specific assay based on stimulation of FSH release in vitro from pituitaries of male rats (2, 9). FSH released into the incubation medium was measured by the method of Steelman and Pohley (17).

RESULTS AND DISCUSSION

The purified LH-RH induced LH release in vivo in assay rats at doses of 10 - 15 ng (6). Significant stimulation of FSH secretion from rat pituitary tissue in vitro was obtained with doses of 100 ng of this material (9). Similarly, FSH release was also augmented after in vivo administration to rats and humans (6, 10). Further purification of this material by partition chromatography on columns of Sephadex G-25 in 10 different solvent systems did not result in separation of LH-RH and FSH-RH activities. The summary of experiments with enzymes illustrated in Table I shows that exposure of LH-RH/FSH-RH to pH 2, 5 and 8.1 did not alter the biological activity. Similarly, trypsin and pepsin did not affect the LH-RH or FSH-RH activity. However several other endopeptidases such as chymotrypsin, subtilisin and papain abolished both the LH-RH activity and FSH-RH activity. LH-RH was also inactivated by leucine aminopeptidase. In several experiments aminopeptidase M appeared

TABLE I

EFFECT OF VARIOUS PROTEOLYTIC ENZYMES ON THE BIOLOGICAL ACTIVITY OF LH-RH AND FSH-RH

Treatment	LH-RH Activity	FSH-RH Activity
pH 8.1 buffer	No effect	No effect
pH 2 buffer	No effect	No effect
pH 5.1 buffer	No effect	No effect
Trypsin	No effect	No effect
Pepsin	No effect	No effect
Chymotrypsin	Inactivation	Inactivation
Subtilisin	Inactivation	Inactivation
Papain	Inactivation	Inactivation
Aminopeptidase M	Inactivation	No effect
Leucine aminopeptidase	Inactivation	Not tested
Carboxypeptidase A	No effect	No effect
Carboxypeptidase B	No effect	No effect
Carboxypeptidase A & B	No effect	No effect

E:S = 1:10 for all enzymes. Incubated at 37° for 20 hours except for carboxypeptidase A & B which was 48 hours. When inactivation was denoted it was essentially complete.

to inactivate LH-RH but not FSH-RH activity. The differences in the relative resistance of FSH-RH to inactivation by aminopeptidase M, as compared to LH-RH, can be perhaps explained by differences in the assay procedures. FSH-RH was measured by the stimulation of release of FSH over a 6 hr incubation period, in a system in which the residual hormone could still, perhaps,

exert small accumulative effects. On the other hand, the activity of LH-RH was assessed by an acute in vivo test based on the measurement of serum LH levels 20 min after the administration of LH-RH. None of the enzymes per se had any effect on LH release or FSH release. It is possible to conclude from the results of the experiments with proteolytic enzymes that LH-RH and FSH-RH have peptide linkages, the splitting of which abolishes biological activity.

The effect of treatment with various reagents on the biological activity of LH-RH and FSH-RH is illustrated in Table II. None of the chemical reagents, at the concentrations found in aliquots used for bioassays, had any effect on LH release in vivo or FSH release in vitro. The lack of effect of

TABLE II

EFFECT OF VARIOUS CHEMICAL TREATMENTS ON BIOLOGICAL ACTIVITY OF LH-RH AND FSH-RH

Treatment	LH-RH	FSH-RH
HCl 0.9 M, 60 min., 100° C	Inactivation	Inactivation
N-bromosuccinimide	Inactivation	Inactivation
Diazotized sulfanilic acid	Inactivation	Inactivation
Sodium carbonate, 5%	No effect	No effect
Nitrous acid	No effect	No effect
Ninhydrin	Inactivation	Inactivation
2-hydroxy-5-nitrobenzyl bromide	No effect	No effect
Sodium borohydride	No effect	Not tested

Editor's Note: Numerical data, too lengthy to be included here, have been provided to support the results in the Tables.

sodium borohydride suggests that the carboxyl terminus may be blocked, in agreement with the results of experiments utilizing carboxypeptidase A and B. It is of interest that nitrous acid did not affect LH-RH or FSH-RH activity. Loss of LH-RH activity on mild acid hydrolysis with 0.9 N HCl is in agreement with earlier results obtained by one of us and suggests splitting of an amide group, an acetyl group or one of the peptide linkages (11). Treatment with N-bromosuccinimide and diazotized sulfanilic acid completely abolished the LH-RH activity. Since 2-hydroxy-5-nitrobenzyl bromide did not inactivate LH-RH, these results indicate that the histidine or tyrosine residue, but not tryptophan, is necessary for biological activity.

Other properties of LH-RH and FSH-RH could be deduced from their behavior during the purification (5, 6). Some of the techniques used were identical to those employed for the isolation of TRH (18). The molecular weight of LH-RH and FSH-RH was estimated to be 1000 - 1500 (1-5), but recent results indicate that this may have to be revised to below 600 (11). Recently the molecular structure of porcine TRH was shown to be (pyro)glu-his-pro amide (19). The elution curves from Sephadex G-25 indeed suggest that the molecular weight of LH-RH/FSH-RH is of the same order of magnitude as that of TRH. During the purification procedures, LH-RH and FSH-RH is more strongly retained on CM-cellulose than TRH (5-7, 18). Free-flow electrophoresis also suggest a more basic isoelectric point or a higher number of positive charges in LH-RH/FSH-RH as compared to TRH (6, 18).

The results reported here indicate that LH-RH and FSH-RH (is) are most likely short chain basic polypeptide(s). However they do not disprove the hypothesis (11) that one molecule is responsible for the release of both FSH and LH.

REFERENCES

1. Schally, A.V., Arimura, A., Bowers, C.Y., Kastin, A.J., Sawano, S., and Redding, T.W.: Recent Progr. Hormone Res. 24: 497 (1968) Academic Press.
2. Mittler, J.C. and Meites, J.: Endocrinology 78: 500, 1966.
3. Guillemin, R., Jutisz, M. and Sakiz, E.: C.R. Acad. Sci. Paris 256: 504, 1963.
4. Fawcett, C.P., Reed, M., Charlton, H.M. and Harris, G.W.: Biochem. J. 106: 229, 1968.
5. Schally, A.V., Bowers, C.Y., White, W.F. and Cohen, A.I.: Endocrinology 81: 77, 1967.
6. Schally, A.V., Arimura, A., Kastin, A.J., Reeves, J.J., Bowers, C.Y., Baba, Y. and White, W.F.: Proceedings of the 1970 Mossbach Kolloquium on Animal and Human Reproduction, Springer-Verlag, Berlin, 1971.
7. Schally, A.V., Arimura, A., Bowers, C.Y., Kastin, A.J., Wakabayashi, I., Mittler, J.C., Redding, T.W. and Pizzolato, P.: J. Clin. Endocr. Metab. 31: 1291, 1970.
8. Fawcett, C.P., In: (J. Meites, ed.) Hypophysiotropic Hormones of the Hypothalamus: Assay and Chemistry, The Williams and Wilkins Co., Baltimore, 1970, p. 242.
9. Schally, A.V., Mittler, J.C. and White, W.F.: Endocrinology 86: 903, 1970.
10. Kastin, A.J., Schally, A.V., Gual, C., Midgley, A.R., Jr., Bowers, C.Y. and Gomez-Perez, E.: Am. J. Obstet. Gynec. 108: 177, 1970.
11. White, W.F., In: (J. Meites, ed.) Hypophysiotropic Hormones of the Hypothalamus: Assay and Chemistry, The Williams and Wilkins Co., Baltimore, 1970, p. 248.
12. Bailey, J.L., Techniques in protein chemistry, American Elsevier Publishing Company, Inc., New York, 1962, p. 22.
13. Shaltiel, S. and Patchornik, A.: J. Amer. Chem. Soc. 85: 2799, 1963.
14. Koshland, D.E., Jr., Karkhanis, Y.D. and Latham, H.G., J. Am. Chem. Soc. 85: 1448, 1964.
15. Ramirez, V.D. and McCann, S.M.: Endocrinology 73: 193, 1963.
16. Niswender, G.D., Midgley, A.R., Jr., Monroe, S.E., Reichert, L.E., Jr.: Proc. Soc. Exp. Biol. Med. 128, 807, 1968.
17. Steelman, S.L. and Pohley, F.: Endocrinology 53: 604, 1953.
18. Schally, A.V., Redding, T.W., Bowers, C.Y. and Barrett, J.F.: J. Biol. Chem. 244: 4077, 1969.
19. Nair, R.M.G., Barrett, J.F., Bowers, C.Y. and Schally, A.V.: Biochemistry 9: 1103, 1970.