ON THE STRUCTURE OF THE HYPOTHALAMIC LUTEINIZING RELEASING HORMONE. EVIDENCE FOR THE PRESENCE OF ARGININE, TYROSINE, AND TRYPTOPHAN BY INACTIVATION

by

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Summary

Structural data on bovine and porcine luteinizing releasing hormone (LRH) of the hypothalamus were obtained by chemical and enzymic inactivations and bioassays. Trypsin and phenylglyoxal inactivated bovine LRH indicating an Arg moiety. Tyrosinase and tetranitromethane inactivated bovine LRH indicating a Tyr moiety. N-Ethylmaleimide and Raney nickel did not inactivate bovine LRH indicating the absence of -SH and S, respectively. 2-Hydroxy-5-nitrobenzyl bromide and its dimethyl sulfonium salt inactivated bovine and porcine LRH indicating a Trp moiety in contrast to previous negative data for Trp.

LRH now appears to be a decapeptide rather than a nonapeptide as reported by others.

Gregory et al. (1) and Schally et al. (2) reported that the luteinizing releasing hormone (LRH) of the hypothalamus is probably a basic peptide. Currie et al. (3) reported the failure of the ninhydrin reagent to inactivate bovine LRH, therefore, the basicity of LRH does not seem to be due to the presence of a free amino group(s) in the molecule. This observation is also supported by other results showing that both ovine and bovine LRH have a blocked N-terminus in the form of a pGlu-moiety; Burgus et al. (4); Currie et al. (3). There are contradictory statements concerning the inactivation of LRH by trypsin. Fawcett et al. (5) and McCann and Ramirez (6) have reported that bovine LRH concentrates were inactivated by trypsin. Ovine LRH (1,7) and porcine LRH (8) preparations were also reported to be inactivated by trypsin. In contrast to these results, Schally et al. (2) reported that a porcine LRH preparation was not inactivated by trypsin.

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Inactivation reactions of biological activities constitute a well-known approach to ascertain structural features of unknown active principles. We have examined as broadly as feasible the inactivation reactions of concentrates of bovine LRH.

Materials and Methods

The following steps served as a basis for preparing concentrates of LRH from frozen hypothalami of bovine and porcine origin: lyophilizing, defatting, extraction with absolute methanol, gel filtration on Sephadex G-25 and two filtrations on Bio-Gel P2. Samples of the bovine and porcine LRH concentrates were used in the chemical and enzymic inactivations, and the data are summarized in Tables I and II.

The digestion with trypsin was carried out in 0.1 M ammonium acetate buffer at pH 8.3 for 20 hours. The incubation with tyrosinase was performed in 0.01 M phosphate buffer at pH 6.8. The reaction with phenylglyoxal hydrate was carried out in 0.1 M phosphate buffer at pH 8.0 for two hours. For the treatment with tetranitromethane (TNM), the method of Sokolovsky et al. (9) was used. The reaction with 2-hydroxy-5-nitrobenzylbromide was performed according to Koshland et al. (10). The inactivation with dimethyl(2-hydroxy-5-nitrobenzyl) sulfonium bromide was performed for 20 minutes at pH 4. The reaction with N-ethylmaleimide was carried out as suggested by Leslie et al. (11). The treatment with Raney nickel was made in ethanol for ten minutes at 25° and 90°. For each inactivation, control experiments were performed by omitting the LRH concentrate and assaying the reaction reagents, and by assaying the LRH concentrate without exposure to the inactivation conditions.

The bioassays for LRH activity were performed by the method of Ramirez and McCann (12). Adult Sprague-Dawley female rats were used six weeks to three months after ovariectomy. The rats were injected subcutaneously with 50 μg of estradiolbenzoate and 25 mg of progesterone, dissolved in sesame oil, 72 hours before the assay. Under ether anesthesia, blood was collected from the jugular vein, and the test sample was injected into this vein; in fifteen minutes, blood was again collected. Each sample was assayed in two rats. Serum assays for LH were performed in duplicate by the double antibody radioimmuno assay of Niswender et al. (13) using antiovine LH serum and ovine LH-^{131}I. The results are expressed in terms of mμg/ml of LER-1240-2-0.60 NIH-LH-SI units/mg. The results of the inactivations were determined by comparison of LH levels before and after injection of the test samples.

Results and Discussion

Exposure of bovine concentrates of LRH to trypsin resulted in complete loss

of the hormonal activity. Trypsin preferentially cleaves peptide bonds on the carboxyl side of arginine and/or lysine. Consequently, these inactivations indicate that arginine is present as a moiety in bovine LRH, since our inactivation data with ninhydrin were negative and did not show the presence of a lysine moiety (3).

Phenylglyoxal appears to be the most recent and a satisfactory chemical reagent for the location of arginine in peptides (14). Phenylglyoxal reacts with an arginine moiety and with an α-amino group at comparable rates. However, an α-amino group is not present since pGlu is the N-terminus in bovine LRH (3). After treatment of concentrates of bovine LRH with phenylglyoxal hydrate, LRH activity was lost. This inactivation provided evidence for the presence of an arginine moiety in LRH.

The inactivation obtained when bovine and also porcine LRH preparations were allowed to react with the Pauly reagent (2,3) showed that at least one of the aromatic amino acids, tyrosine, histidine, or tryptophan, is a moiety, in LRH.

Tyrosinase from mushroom has been shown to oxidize the tyrosine moiety in peptides (15). Exposure of bovine LRH to tyrosinase resulted in loss of the hormonal activity and indicates the presence of a tyrosine moiety. This result is further supported by the inactivation obtained by tetranitromethane (TNM). This reagent has been found to be appropriate for the nitration of tyrosine moieties in proteins (9). Even though some reports have appeared (16,17) which show that under certain conditions TNM can react with methionine and tryptophan moieties, this reagent has proved to be selective for locating tyrosine moieties in proteins (14).

Schally et al. (2) reported that 2-hydroxy-5-nitrobenzylbromide (HNB or Koshland's reagent) did not inactivate a preparation of porcine LRH. This reagent appeared to be highly selective for tryptophan moieties in proteins (14). We have used a recently introduced improvement by Horton and Tucher (18) of Koshland's reagent. In the form of the dimethyl sulfonium salt, 2-hydroxy-5-nitrobenzylbromide has some advantages since it is water soluble and also quite stable in aqueous media. Treatment of bovine and porcine concentrates of LRH with this new reagent and with Koshland's reagent completely abolished the LRH activity.

The inactivation of bovine and porcine concentrates of LRH by Koshland's reagent and by dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide indicate that a tryptophan moiety is present. There has been no previous report in the literature on the positive presence of a tryptophan moiety in LRH of any mammalian ori-

TABLE I. INACTIVATIONS OF CONCENTRATES OF BOVINE LRH

Inactivation Reagent	Fragment Equiv./ Dose	LRH[a] Concentrate Control		Inactivation[b] Reaction Material		Reagent[c] Control	
		mμg LH/ml serum					
		before	after	before	after	before	after
Trypsin	3.0			4.0	4.0		
				5.8	5.6		
		4.0	17.0	5.0	4.0		
		5.0	46.0	6.4	5.8		
				4.0	4.0		
				4.0	4.0		
	3.5	2.0	35.0	3.25	3.5		
		2.2	11.5	<2.0	<2.0		
Phenylglyoxal	1.8	<4.0	110.0	<4.0	5.0	4.0	4.0
		<4.0	12.0	<4.0	4.0	-	-
	3.7	<4.0	54.0	<4.0	4.0	<4.0	4.0
		5.0	64.0	<4.0	4.0	<4.0	4.0
Tyrosinase	4.5	4.0	35.0	<4.0	<4.0	<4.0	<4.0
		4.0	55.0	<4.0	8.0	<4.0	6.0
	3.1			6.0	5.0		
		4.0	30.0	4.0	4.0		
		4.0	17.4	5.0	8.0		
				4.0	4.0		
Tetranitromethane	1.5	<4.0	24.0	<4.0	4.0	<4.0	4.0
		<4.0	23.0	<4.0	4.0	<4.0	4.0
	3.1	4.0	30.0	8.0	10.0	6.0	7.0
		4.0	17.4	4.0	10.0	4.0	8.0
	3.7	6.0	31.0	<4.0	<4.0	<4.0	<4.0
		5.0	56.0	<4.0	<4.0	<4.0	<4.0
Dimethyl(2-hydroxy-5-nitro benzyl)sulfonium bromide	2.0	<4.0	62.0	4.0	4.0	4.0	4.0
		<4.0	15.0	4.0	4.0	4.0	4.0
	1.7	4.0	12.0	4.4	4.8	4.0	<4.0
		4.0	15.8	<4.0	6.4	4.6	4.0
	4.5	4.0	112.8	<4.0	<4.0	<4.0	4.0
		4.0	112.8	4.0	4.0	4.0	6.0
	4.5	2.0	91.4	3.6	4.0	4.0	4.0
		2.0	101.4	2.6	2.6	4.0	6.0
2-Hydroxy-5-nitro-benzyl bromide	3.5	5.8	80.0	4.0	3.8	2.8	3.0
		5.8	142.8	3.8	4.0	2.8	2.5
N-Ethylmaleimide	4.5	<4.0	112.0	<4.0	40.0	<4.0	<4.0
		<4.0	112.0	<4.0	34.0	<4.0	<4.0
	5.0	2.0	91.4	4.0	142.8		
		2.0	101.4	10.0	142.8		

a) LRH preparation and the reagents (e.g. buffer etc.) except reactive species.
b) LRH preparation and all the reagents necessary for reactions.
c) All the reagents except the LRH concentrate.

gin and negative evidence for tryptophan was reported for porcine LRH (2).

Schally et al. (19) reported that "the nonapeptide isolated from porcine hy-

TABLE II. INACTIVATIONS OF CONCENTRATES OF PORCINE LRH

Inactivation Reagent	Fragment Equiv./ Dose	LRH[a] Concentrate Control		Inactivation[b] Reaction Material	
		mμg LH/ml serum			
		before	after	before	after
2-Hydroxy-5-nitrobenzyl bromide	0.6	4.0	126.0	4.0	4.0
		<4.0	186.0	4.0	4.4
Dimethyl (2-hydroxy-5-nitro benzyl)sulfonium bromide	0.6	4.0	126.0	6.0	8.0
		<4.0	186.0	4.0	4.0

a) LRH preparation and the reagents (e.g. buffer etc.) except reactive species.
b) LRH preparation and all the reagents necessary for reactions.

pothalami" has both LH-RH and FSH-RH activity, and yielded 1 His; 1 Arg; 1 Ser; 1 Glu; 1 Pro; 2 Gly; 1 Leu, and 1 Tyr by acid hydrolysis.

pGlu, Arg, and Tyr are apparently present in bovine LRH according to our inactivation data, and pGlu, Arg, Tyr, and six other amino acids are apparently present in porcine LRH (19). This information in conjunction with our data on Trp indicate that LRH is a decapeptide rather than a nonapeptide as expressed by Schally et al. (19).

Both HNB and TNM can also react with cysteine (9,18) and it was, therefore, important to test for the presence of this amino acid. The nucleophilic addition of sulfhydryl groups to the double bond of N-alkylmaleimides has been employed in structural studies of proteins (14). Bovine LRH concentrates were still active after exposure to N-ethylmaleimide indicating that no sulfhydryl group is present in LRH. Exposure of concentrates of LRH to Raney nickel for possible hydrogenolysis of sulfur-containing bonds did not result in loss of hormonal activity. Again, the absence of sulfur in LRH is indicated.

In a companion paper by Chang et al. (20), the discovery of a new synthetic tetrapeptide having luteinizing releasing hormone (LRH) activity is described. The synthesis of the six possible tetrapeptides based on these and our previous inactivation studies on concentrates of LRH, followed by bioassays, revealed that only one of the six sequences, pGlu-Tyr-Arg-Trp-NH_2 exhibits the hormonal activity of LRH. The structural interpretations drawn from these inactivation studies of LRH are supported by this discovery of hormonal activity of synthetic pGlu-Tyr-Arg-Trp-NH_2.

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