ACTIVITY OF A NEW SYNTHETIC TETRAPEPTIDE IN HYPOTHALAMIC LUTEINIZING AND FOLLICLE STIMULATING RELEASING HORMONE ASSAY SYSTEMS

by

Cyril Y. Bowers

Tulane University
School of Medicine
New Orleans, Louisiana 70112

and

Jaw-Kang Chang, Hans Sievertsson, Conny Bogentoft,
Bruce L. Currie, and Karl Folkers*

Institute for Biomedical Research
The University of Texas at Austin
Austin, Texas 78712

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Summary

Synthetic pGlu-Tyr-Arg-Trp-NH_2 released, *in vivo*, the luteinizing hormone (LH) from the pituitary gland, but there was no release of the follicle stimulating hormone (FSH) or thyrotropin (TSH). All four amino acids of this peptide seem to be included in the apparent ten amino acids of LRH. Together, this tetrapeptide and the thyrotropin releasing hormone released LH and TSH. This tetrapeptide also released LH, *in vitro*, indicating the pituitary as the site of action of this peptide. This relatively potent tetrapeptide is not LRH, and its failure to release FSH may indicate a "separation" of the functionalities of the molecule of LRH which release both LH and FSH if, indeed, one releasing hormone controls both LH and FSH.

Studies by Currie *et al*. (1) on the structure of bovine LRH by specific chemical and enzymatic inactivations of concentrates of the hormone as determined by *in vivo* assays led to the finding that bovine LRH apparently has a N-terminal pGlu-moiety, a His or Tyr or other Pauly-reactive amino acid(s) and no free amino group.

Continuing these studies on the structure of both bovine and porcine LRH, Bogentoft *et al*. (2) interpreted previous and new inactivation experiments on LRH, particularly of bovine origin, on the basis that bovine LRH appears to contain pGlu, Tyr, Arg, and Trp moieties; also, it was found that porcine LRH appears to contain a Trp moiety in contrast to the negative results on Trp by Schally *et al*. (3).

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Since these inactivation studies on LRH with relatively specific chemical reagents and enzymes had indicated the presence of the four amino acids, pGlu, Arg, Trp, and Tyr, in the molecule of the hormone, it was evident that these four amino acids could be the only ones in LRH or that there could also be other amino acids present in the hormone which are not possible to detect by these types of inactivation methods. Since the thyrotropin releasing hormone is a tripeptide, pGlu-His-Pro-NH_2, and since the present knowledge on the structure of LRH points to a molecule of relatively low molecular weight, it was considered expedient for several reasons to synthesize the six possible tetrapeptides consisting of pGlu, Tyr, Trp, and Arg. If LRH has more amino acids than four, then it was believed that data on the bioassays, *in vivo*, of the six sequences of the four amino acids would ultimately contribute to the knowledge of structure-activity relationships on LRH as had been the situation on the structure-activity relationships for TRH (4). The evolving overall knowledge on structure-activity relationships of several of these hypothalamic hormones, which is of such promising medical importance, could uniquely advance the progress on the remaining hormones of the hypothalamus.

Chang *et al*. (5) synthesized the six possible tetrapeptides of pGlu, Tyr, Trp, and Arg and they were bioassayed *in vivo* for LRH activity. It was found that one tetrapeptide, pGlu-Tyr-Arg-Trp-NH_2, significantly increased the release of the luteinizing hormone. The other five possible tetrapeptides of these four amino acids have no LRH activity at comparable dose levels.

To our knowledge, a synthetic peptide having LRH activity has not yet been reported in the literature. The availability of this synthetic tetrapeptide is timely, and it has some advantages of current importance in connection with and separate from that of LRH itself. Consequently, this tetrapeptide is being biologically evaluated in a number of assay systems, and we now make our first detailed report on its hormonal activities in addition to the brief account given by Chang *et al*. (5).

In principle, to determine whether a peptide has the activity of a hypothalamic releasing hormone, it is of importance to determine the activity *in*

vitro as well as *in vivo*. Activity in the *in vitro* system of the pituitary indicates the pituitary as the site of action. Activity *in vivo* indicates the ultimate response, but not necessarily mediation by the pituitary. Activity in which only one of the anterior pituitary hormones is primarily released indicates the specificity of hormonal action as does the sensitivity of the response in specially designed biological assay systems. By use of these types of assay systems, it has been possible to purify the hypothalamic hormone(s), LRH and FRH from tissue fragments of the hypothalamus of pigs, sheep, cattle, and humans (6-12). All the research in this field by many investigators has led to the working hypothesis that there may be one hypothalamic neurohormone which releases one pituitary hormone of the anterior lobe. An exception to this hypothesis is the proposal by White (13) that there may be a hypothalamic "monotropic" control for the gonadatropins, LH and FSH. In 1971, Schally *et al*. (14) reported that the porcine "peptide-substance", which is active at nanogram dose-levels, was "essentially pure" and released both LH and FSH. Schally *et al*. (3) also reported that the same chemical and enzymic procedures when applied to their porcine LRH inactivated the hormonal release of both LH and FSH. Kastin *et al*. (15) administered highly purified porcine LRH to humans and observed a release of both LH and FSH.

The release of two anterior pituitary hormones, LH and FSH, by one hypothalamic neurohormone has seemed inherently improbable to some investigators, and this exception to the concept of one hypothalamic hormone releasing one anterior pituitary hormone awaits the availability of conclusive evidence for the "monotropic" release of FSH and LH.

In the meantime, it is pertinent to assay the synthetic pGlu-Tyr-Arg-Trp-NH_2 not only for the release of LH, but also the release of FSH. If one hypothalamic hormone does indeed release both LH and FSH, it is conceivable that a structurally modified form of the hypothalamic hormone might also release both LH and FSH, but the bioassays described herein reveal that this dual release is not observed for the synthetic tetrapeptide, and to this extent does not sup-

port the concept of "monotropic" control. However, this lack of support is only interim evidence, since this synthetic tetrapeptide may represent "separation" of the functionalities of the molecule of LRH which releases both LH and FSH.

Methods

The *in vivo* studies were performed in Adult Sprague-Dawley female rats six weeks to three months after ovariectomy. The rats were subcutaneously injected with 50 μg of estradiol benzoate and 25 mg of progesterone, dissolved in sesame oil, 72 hours before injection of test samples according to the method of Ramirez and McCann (16). Under ether anesthesia, blood was collected from the jugular vein and test samples were injected into the same vein. Serum assays for LH and FSH were performed in duplicate by the double antibody radioimmuno assay of Niswender *et al.* (17) and the NIAMD method of Parlow (18). The LH results are expressed in terms of mμg/ml of LER-1240-2-0.60 NIH-LH-SI units/mg, and the FSH results are expressed in terms of NIAMD-Rat FSH-RP-1 (2.1 X NIH-FSH-S-1). TSH was assayed by the T_3-TSH method in mice (19). The TSH results are expressed in terms of the bovine TSH preparation Thytropar (Armour Pharmaceutical Co.). The *in vitro* studies were performed at 37° C in air as described (19,20) using 1.5 ml of Ringers-lactate solution per beaker. Each vessel also contained 1 mg glucose per ml and 0.1% bovine serum albumin. There were two to four 30-minute pre-incubation periods.

Results

As detailed in Table I, the levels of LH rose slightly after a single intravenous injection of 50 μg of pGlu-Tyr-Arg-Trp-NH_2. As the dose was increased, the levels of LH, but not FSH or TSH, were 30 to 40 times higher than the level at zero time.

The data in Table II show that the specificity of action was again demonstrated when it was found that pGlu-Tyr-Arg-Trp-NH_2 raised serum levels of LH but not TSH while pGlu-His-Pro-NH_2 (TRH) raised serum levels of TSH but not LH. When the compounds were administered together, serum levels of both LH and TSH

TABLE I. ACTIVITY OF pGLU-TYR-ARG-TRP-NH_2 IN VIVO

Rat	Dose μg	LH 0	LH +15 min.	FSH 0	FSH +15 min.	TSH 0	TSH +15 min.
		mμg/ml		μg/ml		^{125}I Δcpm rise	
1	50	2.5	12.0	1.3	1.2	-	-
2	50	5.7	8.5	1.1	1.1	-	-
3	200	3.3	120.0	0.9	1.1	231	292
4	200	4.2	>142.0	2.0	1.6	64	64
5	600	4.2	>142.0	1.2	1.3	131	76
6	600	6.5	>142.0	1.6	1.8	-	-

TABLE II. IN VIVO EFFECT OF pGLU-TYR-ARG-TRP-NH_2 (I) AND pGLU-HIS-PRO-NH_2 (II) ON RELEASE OF LH AND TSH

	Dose μg	0	+15	+30	+60	+120	min.	0	+15	+30	+60	+120
		mμg LH/ml serum						mu TSH/100 ml serum				
I	500	5	140	126	32	14		<30	<20	<30	<30	<30
II	10	4	5	6	7	7		<30	58	44	<30	<30
II	100	13	10	9	10	11		<30	180	140	34	<30
I + II	500 + 10	5	130	61	27	23		<30	50	44	36	34
I + II	500 + 100	4	22	15	10	7		<30	48	30	38	34

I and II were injected iv alone and together after collection of blood at 0 time. Recorded are the levels of LH and TSH measured in the same samples of serum collected at various times after injection of the compounds.

TABLE III. SPECIFICITY OF ACTION, DURATION OF EFFECT, AND RESPONSE BY DIFFERENT ROUTES OF ADMINISTRATION OF pGLU-TYR-ARG-TRP-NH_2 IN VIVO

Rat #	Min.	LH mμg/ml	FSH μg/ml	TSH Δcpm[1]	Rat #	LH mμg/ml	FSH μg/ml	TSH Δcpm[1]
1					2			
	0	2.8	1.8	960		2.0	2.0	394
	+15	85.0	1.7	108		103.0	1.9	142
	+30	75.0	1.7	265		83.0	1.9	97
	+60	40.0	1.6	130		41.0	2.2	43
3					4			
	0	2.0	2.3	137		2.0	1.7	233
	+15	2.2	2.0	189		2.1	1.5	213
	+30	2.2	1.8	92		3.1	1.4	189
	+60	4.9	2.0	38		6.5	1.4	84

500 μg of pGlu-Tyr-Arg-Trp-NH_2 was administered iv at 0 time to rats 1 and 2 and sc to rats 3 and 4.
[1] ^{125}I Δcpm rise in blood levels in TSH assay mice.

rose. The rise of LH and TSH were less when 100 μg (a very large dose) of pGlu-His-Pro-NH_2 was administered in combination with pGlu-Tyr-Arg-Trp-NH_2 than when they were administered alone. The significance of these interesting latter findings are unknown at this time and, thus, they are being studied in more detail.

The results in Table III show that serum levels of LH were 30 to 50 times higher fifteen minutes after a single intravenous injection of pGlu-Tyr-Arg-Trp-NH_2, and the levels were still elevated after thirty and sixty minutes. In contrast, there was no change in the levels of FSH while the levels of TSH appeared to decrease. When pGlu-Tyr-Arg-Trp-NH_2 was administered subcutaneously, there was only a very slight rise in the levels of LH after sixty minutes, but there was no rise in the levels of FSH or TSH.

The data in Table IV indicate that pGlu-Tyr-Arg-Trp-NH_2 also stimulates

TABLE IV. EFFECT OF pGLU-TYR-ARG-TRP-NH_2 (TP) ON RELEASE OF LH AND FSH FROM PITUITARY IN VITRO

Exp	Hour	TP added to E μg	C mμg LH/ml medium	E mμg LH/ml medium	C μg FSH/ml medium	E μg FSH/ml medium
I-A	1	100	410	550	7.0	7.3
	2	100	300	825	4.8	5.3
	3	100	375	925	5.1	6.0
I-B	1	100	300	650	6.8	6.5
	2	100	290	1280	4.8	6.3
	3	100	382	712	5.3	6.5
II-A	1	50	134	302	5.5	6.6
	1	50	156	280	5.2	5.1
II-B	1	.5 SME	157	375	6.2	10.0

Each control (C) and experimental (E) beaker contained 1.5ml Ringers-lactate medium and 2 corresponding halves of anterior pituitary glands removed from ovariectomized (3 months) adult rats pretreated with estradiol and progesterone (72 hr). After two (Exp I-A and B) or four (Exp II-A and B) 30 minute preincubation periods, the medium was removed for assay of LH and FSH and fresh medium was added each hour for three hours (Exp I) or for one hour (Exp II). After preincubation the tetrapeptide or a partially purified LRH extract equivalent to 0.5 bovine hypothalamic stalk median eminence fragment (SME) was added to the medium of the E beaker at the beginning of each hourly incubation period.

the release of LH but not FSH, in vitro, and it appears that the pituitary is the site of action of this tetrapeptide. When a partially purified bovine stalk median eminence extract was added in vitro, there was increased release of both LH and FSH.

These biological assay data demonstrate that the new synthetic tetrapeptide, pGlu-Tyr-Arg-Trp-NH_2, releases LH, in vivo, in the rat and acts directly upon cells of the pituitary as evidenced by the in vitro data. The hormonal activity of this synthetic tetrapeptide in conjunction with the inactivation data (1,2) on LRH, indicate the presence of the moieties of pGlu, Arg, Trp, and Tyr in the structure of LRH, and as evaluated in our other papers (2,5), LRH appears to be a decapeptide. The finding that this synthetic tetrapeptide does not also release FSH indicates that (a) one hypothalamic hormone releases LH and another releases FSH or (b) the structural difference between pGlu-Tyr-Arg-Trp-NH_2 and natural LRH is such that the functionalities of the molecule which release LH and those which release FSH have been "separated".

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REFERENCES

1. Currie, B.L., Sievertsson, H., Bogentoft, C., Chang, J.K., Folkers, K., Bowers, C.Y., and Doolittle, R.F., Biochem. Biophys. Res. Commun., 42, 1180 (1971).
2. Bogentoft, C., Currie, B.L., Sievertsson, H., Chang, J.K., Folkers, K., and Bowers, C.Y., Biochem. Biophys. Res. Commun., 43, (1971).
3. Schally, A.V., Baba, Y., Arimura, A., Redding, T.W., and White, W.F., Biochem. Biophys. Res. Commun., 42, 50 (1971).
4. Chang, J.K., Sievertsson, H., Currie, B.L., Folkers, K., and Bowers, C.Y., J. Med. Chem., 14, (1971), (in press).
5. Chang, J.K., Sievertsson, H., Bogentoft, C., Currie, B.L., Folkers, K., and Bowers, C.Y., Biochem. Biophys. Res. Commun., 43, (1971).
6. Schally, A.V., Arimura, A., Bowers, C.Y., Kastin, A.J., Sawano, S., and Redding, T.W., Rec. Progr. Hormone Res., 24, 497 (1968).
7. Mittler, J.C. and Meites, J., Endocrinology, 78, 500 (1968).
8. Guillemin, R., Jutisz, M., and Sakiz, E., C. R. Acad. Sci. Paris, 256, 504 (1963).
9. Fawcett, C.P., Reed, M., Charlton, H.M., and Harris, G.W., Biochem. J., 106, 229 (1968).

10. Schally, A.V., Bowers, C.Y., White, W.F., and Cohen, A.I., Endocrinology, 81, 77 (1967).
11. Schally, A.V., Arimura, A., Kastin, A.J., Reeves, J.J., Bowers, C.Y., and Baba, Y., In Mammalian Reproduction, H. Gibian and E.J. Plotz, eds., p. 45, 1970, Springer-Verlag, Berlin.
12. 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).
13. White, W.F., In Hypophysiotropic Hormones of the Hypothalamus, J. Meites, ed., p. 248, 1970, The Williams and Wilkins Co., Baltimore.
14. Schally, A.V., Arimura, A., Baba, Y., Nair, R.M.G., Matsuo, H., Redding, T.W., Debeljuk, L., and White, W.F., Biochem. Biophys. Res. Commun., 43, 393 (1971).
15. Kastin, A.J., Schally, A.V., Gual, C., Midgley, Jr., A.R., Bowers, C.Y., and Gomez-Perez, E., Am. J. Obstet. Gynec., 108, 177 (1970).
16. Ramirez, V.D. and McCann, S.M., Endocrinology, 73, 193, (1963).
17. Niswender, G.D., Midgley, Jr., A.R., Monroe, S.E., and Reichert, Jr., L.E., Proc. Soc. Exp. Biol. Med., 128, 807 (1968).
18. Parlow, A.F., Daane, T.A., and Schally, A.V., Abstracts 51st, Meeting of the Endocrine Society, p. 83, New York, 1969.
19. Bowers, C.Y., Schally, A.V., Enzmann, F., Bøler, J., and Folkers, K., Endocrinology, 86, 1143 (1970).
20. Bowers, C.Y. and Robison, G.A., In 1970 New York Academy of Sciences Conference on Cyclic AMP and Cell Function, (in press).