Vol. 81, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1978 Pages 680-683

ISOLATION OF PYROGLUTAMIC ACID FROM HYPOTHALAMIC TISSUE AND SIGNIFICANCE OF ITS INHIBITION OF PROLACTIN RELEASE

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Received February 13,1978

SUMMARY

Extensive fractionation of extractives of porcine hypothalamic tissue was scaled-up with the guidance of bioassays including the inhibition of prolactin release, because previously ca. <100 ng of fraction, which could have been essentially pure, was active at ca. <5 ng, to inhibit prolactin. Ultimately, an active entity was isolated and characterized as pyroglutamic acid. This amino acid was newly found to inhibit the release of prolactin at μg-levels. The presence and activity of pyroglumatic acid during fractionation for the presumed authentic prolactin inhibiting factor (PIF) was an unknown difficulty, which now may interest others working with PIF extracts and fractions. This difficulty may now be circumvented.

INTRODUCTION

The presence of free pyroglutamic acid (<GluOH) in nature has long been known. Wilk and Orlowski (1), by the application of a gas-liquid chromatographic procedure, found significant amounts of free <GluOH in body fluids (urine and crebral spinal fluid) and tissues. They stated that the levels could not be derived from the non-enzymatic cyclization of glutamine (1,2). <GluOH (5-oxoproline) occurs naturally in tissue as the result of the reaction of γ-glutamylcyclotranferase upon di-γ-glutamyl amino acids (Meister and Tate, 3). Auditore and Wade (4) reported the isolation of large amounts of <GluOH, and its butylester, from human brain. They concluded, however, that <GluOH was more likely an artifact formed from glutamine during isloation.

We have encountered <GluOH during the scale-up to isolate more of our potent fraction of a prolactin inhibiting factor (PIF) (5,6). We have newly

*Peptide Hormone. 121.

0006-291X/78/0812-0680$01.00/0

found that <GluOH inhibits the release of prolactin, although at μg-levels, but can confuse the isolation of hormones which may control the release and inhibition of release of prolactin.

METHODS AND MATERIALS

The sequence of 15 purification steps which yielded <GluOH from porcine hypothalami is in Table I, and the typical bioassay data for inhibition of the release of prelactin are included.

The thin-layer chromatography (TLC) was performed on precoated silica gel 60 tlc plates (thickness, 0.25 mm, purchased from EM Laboratories, Inc.) in two different systems: solvent system A, upper phase of the solvent system, 1% HOAc:n-BuOH:Pyridine (11:5:3); solvent system B, nBuOH:HOAc:H_2O:MeOH (62:12:26:100). The thin-layer electrophoreses (TLE's) were executed on Brinkman precoated cellulose-plastic sheets (thickness, 0.10 mm) at 0.500 KV and 15 mAmps in buffers C, pyridine:HOAc:H_2O (10:100:1000, pH 3.5); and D, pyridine:HOAc:H_2O (100:5:900, pH 6.5). The TLC and TLE results were visualized by the chlorine-tolidine method. Gels for column chromatography were purchased from either Sigma Chemical Co. or Bio-Rad. All solvents were glass distilled. Amino acid analyses were run in a Beckman Model 119 Automatic Amino Acid Analyzer. Field desorption mass spectra were obtained with a Hitachi-Perkin Elmer RMU-7, single focusing mass spectrometer, modified with a field ionization-field desorption ion source, and a SSR Model 1120 ion counting detection system as described (7). The general procedure of the in vitro bioassays and radio-immunoassays have been described (5). Specifically, the pituitaries of 20-day old female rats were incubated in lactated Ringer's solution for 3 hours (P_2 = after 2 hours; and I_3 = incubation after 3 hours or 1 hour of incubation). The medium was changed hourly, and the substances being tested were added at the beginning of I_3. The pituitary hormone released into the medium was measured with the NIAMDD PRL RIA.

<GluOH, purchased from Sigma Chemical Co., was purified by column chromatography on silica gel (Baker), followed by gel filtration on Sephadex G-10.

RESULTS AND DISCUSSION

A product from purification at step 15 was homogeneous on TLC in two different solvent systems, A and B, and on TLE in two different buffer systems, pH 3.5 and pH 6.5. Amino acid analysis of the product showed the presence of glutamic acid with a trace of aspartic acid. Field desorption mass spectra of the same sample revealed an apparent molecular ion of m/e 130. These results, and the facts that the product was ninhydrin negative, but chlorine-tolidine positive, supported the conclusion that the product was essentially pure, and could be <GluOH. Additional physicochemical and biological comparisons of the isolated product and authentic L-<GluOH were then made.

The isolated product and the authentic L-<GluOH showed identical R_f values in TLC's and mixed TLC's in the solvent systems A and B (R_f value in A=0.43; R_f value in B = 0.38). They had identical electrophoretic mobilities in TLE's and mixed TLE's in buffers C and D. They had the same elution volume on identical gel filtration columns of Sephadex G-10. Their field desorption mass spectra were essentially identical. Their activities in vitro for

Table I. Purification Sequence and Typical Bioassay Data on Inhibition of Prolactin Release

Step	Purification	Dose (μg)	ng PRL/ml P_2	ng PRL/ml I_3
1	Extraction of tissue with 2 M HOAc in MeOH			
2	Defatting with methylene chloride			
3	Gel filtration on Bio-Gel P-2	10,000	159	18
4	Partition chromatography on Sephades G-25	1,700	92	18
5	Anion exchange on DEAE-Sephadex	700	145	4
6	Repartition chromatography on Sephadex G-25	480	139	9
7	Gel filtration on LH-20	250	200	12
8	Partition chromatography on Sephadex G-25	400	119	3
9	HPLC, Poragel-PT	500	230	0
10	HPLC, Phenyl-Corasil	150	236	51
11	HPLC, Poragel-PS	160	274	9
12	Cation exchange on SP-Sephadex	700	301	1
13	HPLC, Rechromatography on Poragel-PT	150	172	3
14	Gel filtration on Sephadex G-10	180	235	8
15	Rechromatography on Sephadex G-10	100	158	4

inhibition of prolactic release were comparable, according to Table II, which shows a dose-to-dose comparison of the two products. These findings showed that the isolated product is <GluOH.

We estimate that there is ca. 100 μg of <GluOH in each hypothalamic fragment which has a dry weight of ca. 100 mg. We found that <GluOH can be derived from N-acetylglutamic acid be standing overnight at room temperature in the solvent system 11:5:3, or from glutamine in the solvent of extraction and/or the solvent system 4:1:5 (Table I).

The observation that <GluOH can suppress the in vitro release of prolactin is novel, however, various compounds have been isolated in the search for a prolactin-inhibiting factor (PIF) from hypothalamic extracts by different laboratories. Shaar and Clemens found dopamine (8). They also showed that the PIF activity of hypothalamic extracts closely parallels the content of catecholamine and considered dopamine a physiological PIF (9). Greibrokk et al. reported in 1975 (6) a preparation of ca. <100 ng of a prolactin inhibiting factor from high pressure liquid chromatography (hplc) which showed in vitro activity at ca. <5 ng. The symmetrical single peaks by hplc indicated a degree of purity. <GluOH was readily observable by FDMS on the basis of an intense pseudomolecular ion at m/e 130. This preparation was analyzed by field desorption mass spectrometry (FDMS). The spectra obtained from the preparation, at two final stages of purification, in nine separate analyses revealed no trace of ion intensity at m/e 130 attributable to <GluOH. The spectra obtained

Table II. Biological Comparison of Isolated and Authentic <GluOH to Inhibit, In Vitro, Prolactin Release

Dose (μg)	Isolated <GluOH ng PRL/ml		Authentic <GluOH ng PRL/ml	
	P_2	I_3	P_2	I_3
600	101	1	185	8
400	220	1	134	2
300	107	1	120	3
200	264	14	234	11
150	183	6	167	7
100	158	4	253	22
50	143	93	110	66
25	147	10	96	68
10	126	54	137	86
3	156	115	174	142
1	195	179	191	192

were complex and exhibited numerous ions dispersed over a wide mass-range potentially assignable to the biologically active entity. In a companion experiment, the preparation was hydrolyzed, and the hydrolysate was found to contain amino acids by gas chromatography mass spectrometry (10) which showed the peptidic nature of the preparation. Schally et al., in 1977, isolated gamma-aminobutyric acid (11).

The presence and activity of a high concentration of <GluOH in early and subsequent steps of fractionation of hypothalamic extracts may now be recognized as an interfering substance which can inhibit prolactin release.

ACKNOWLEDGMENT

Appreciation is expressed to the Robert A. Welch Foundation and to the National Cancer Institute, DHEW Grant No. CA-14200-05 and AM 06164 for their respective support of this research.

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