ON THE ISOLATION OF A PROLACTIN INHIBITING FACTOR (HORMONE)

by

Tyge Greibrokk, Jan Hansen, Ronald Knudsen, Yiu-Kuen Lam and Karl Folkers*

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

and

Cyril Y. Bowers

Tulane University
School of Medicine
New Orleans, Louisiana 70112

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SUMMARY

A preparation of ca. <100 ng of a prolactin inhibiting factor was isolated which could be essentially pure, because of symmetrical single peaks by high pressure liquid chromatography. The in vitro activity was at ca. <5 ng which is the highest potency reported by anyone. The paucity of ca. <100 ng/80,000 hypothalami necessitates patience for definitive data on more product from ca. 240,000 to 450,000 hypothalami. Weight was estimated by comparing UV absorption at 220 nm with that of synthetic peptides. This preparation is not a catecholamine by chromatography, and gives new and timely credence to the concept that prolactin secretion is mediated by complex mechanisms including a peptide inhibiting factor and a catecholamine.

INTRODUCTION

The secretion of prolactin by the anterior lobe of the pituitary is generally believed to be controlled by the hypothalamus although the nature of this hormonal control is largely unknown. The participation of a prolactin inhibiting factor (PIF) was independently and concomitantly proposed by Talwalker, Ratner, and Meites (1) and by Pasteels (2).

The role of dopamine in the hypothalamic control of prolactin is not completely understood. Injections of dopamine into the third ventricle and systemic injections of L-dopa were observed to "promote PIF discharge from the hypothalamus" and dopamine was also found to act directly on the pituitary (3,4). The isolation of dopamine from hypothalamic tissue presumably influenced the conclusion that a catecholamine is PIF (5). Other investigators (6,7) viewed dopamine as a neurotransmitter which inhibits

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the release of prolactin by stimulation of release of PIF. It has been suggested (8) that dopamine acts on receptors located on neurons containing PIF. This concept seems consistent with data showing that implants of Pimozide, a dopaminergic receptor-site blocker, in the median eminence but not in the anterior basal hypothalamus, increased plasma prolactin (9). When dopamine was injected into the third ventricle of the brain, the prolactin inhibiting activity was increased in the pituitary stalk plasma of the rat. The prolactin inhibiting activity was attributed to PIF, and its release was believed to be regulated by a dopaminergic mechanism (10).

These citations indicate the existence of PIF and a complex inter-relationship with dopamine or other catecholamines and other factors including cyclic AMP.

Evidence has implicated cyclic AMP as a mediator of the action of dopamine (11). Dibutyryl cyclic AMP (DBC) mimicked the decrease in plasma prolactin induced by dopamine (11). Injections of DBC in the third ventricle of ovariectomized estrogen-treated rats decreased plasma prolactin, but injections into the pituitary were ineffective. DBC reduced plasma prolactin in Pimozide-treated rats indicating action at a post-synaptic site. Intra-ventricular injections of prostaglandin E_1 negated the effect of dopamine but not of DBC (11).

Extracts of hypothalamic tissue have been reported to contain a prolactin releasing entity which is chemically unknown but also may be the thyrotropin releasing hormone (TRH), because TRH not only releases thyro-tropin but also releases prolactin in animals (12-16) and in humans (17-19). Estrogen pre-treated rats (20,21), hypothyroid rats (22) and rat pituitary tumor cells (12) responded to TRH, but untreated male rats were unaffected (21,23). It was reported (23) that intravenous administration of a hypo-thalamic extract increased serum prolactin, but inhibited prolactin in lactating rats; such data indicate a dual control system not affected by TRH. The release of PRL by TRH was depressed by dopamine or L-dopa (20), although previously the opposite effects were indicated (24). Somatostatin inhibited the release of PRL by TRH, but only *in vitro* (25).

Multi-step purification of PIF on a substantial scale of tissue was reported both by Schally *et al.* (26) and by Greibrokk *et al.* (27) in 1974, and by Dupont and Redding (28) in 1975.

Greibrokk *et al.* (27) purified a PIF entity and found that it was in-activated by pronase which seemingly rules out it being a catecholamine. We now report further purification of this PIF-entity, particularly by high pressure liquid chromatography which is a relatively new technique in this field, that yielded a preparation active *in vitro* at *ca.* <5 ng.

METHODS AND BIOASSAYS

Porcine hypothalami were lyophilized, extracted, defatted, and purified by gel filtration, partition chromatography, and ion exchange, as described (27). The PIF-active fractions eluted with 1 M acetic acid from DEAE-Sephadex, were then purified by high pressure liquid chromatography. Four different column packing materials were used: Poragel PT, Bondapak Phenyl-Corasil, Poragel PS, and Poragel PN, all from Waters Associates, Inc. These HPLC purifications were performed on an ALC-202 instrument of Waters Associates, Inc. which was equipped with a 6000 psi pump and two series connected UV-detectors that measured at 220 and at 254 nm. The organic solvents were glass distilled from Burdick and Jackson.

The in vitro bioassays and radioimmunoassays were performed as described (27).

RESULTS AND DISCUSSION

The sequence of purification steps for the isolation of this highly potent preparation showing PIF activity is listed in Table 1.

The chromatographic data stemming from the high pressure liquid chromatographic purifications and the corresponding biological data from the in vitro assays for representative active and side fractions at increasing stages of purity are detailed in Table 2.

As initially reported (27), we continued to achieve purification of PIF on a column of Poragel PT followed by chromatography on Phenyl-Corasil. The separated PIF was then further purified on Poragel PS and Poragel PN and by rechromatography on Phenyl-Corasil and on Poragel PN.

Table 1. Purification Sequence for PIF

1. Extraction with 2 M HOAc in MeOH.
2. Defatting with methylene chloride.
3. Extraction with n-butanol (proceeded with aqueous phase).
4. Gel filtration on Bio-Gel P-2.
5. Rechromatography on Bio-Gel P-2.
6. Partition chromatography (n-butanol-acetic acid-water, 4:1:5) on Sephadex G-25.
7. Partition chromatography (1.0% acetic acid-n-butanol-pyridine, 11:5:3) on Sephadex G-25.
8. Anion exchange on DEAE-Sephadex.
9. HPLC, Poragel PT.
10. HPLC, Bondapak Phenyl-Corasil.
11. HPLC, Poragel PS.
12. HPLC, Poragel PN.
13. HPLC, Rechromatography on Phenyl-Corasil.
14. HPLC, Rechromatography on Poragel PN.

Table 2. HPLC Data and In Vitro Assays

Col. Pack.	Solv. Sys.	Dose µg	Frac.	µg/ml PRL P_2	PRL I_3	LH P_2	LH I_3	FSH P_2	FSH I_3	GH P_2	GH I_3
PT[1]	a	<0.1	AF[5]	126	34	35	18	4246	3331	597	298
			SF[6]	252	226	102	97	5912	4032	1170	993
PC[2]	b	<0.1	AF	229	45	42	62	2685	3877	1047	1102
			SF	338	207	106	97	3834	4335	1112	1150
			SF	258	193	52	48	2363	2090	1994	1691
PS[3]	c	<0.1	AF	358	86	35	45	2360	2165	1692	1678
			AF	454	120	43	62	2275	2377	1366	1146
PN[4]	d	<0.05	AF	173	117	19	23	1443	1457	358	284
			SF	142	128	27	28	880	873	274	144
PC[2]	b	<0.01	SF	281	185	-	-	-	-	-	-
			AF	52	10	23	52	1362	1327	585	620
			SF	64	70	39	42	1361	1239	1200	1176
PN[4]	b	<0.005	SF	75	70	20	25	390	530	630	668
			AF	226	68	39	40	999	1258	1061	948
			SF	154	104	34	38	1266	1055	999	754

[a] 20mM HOAc in 80% MeOH; [b] H_2O; [c] 70% CH_3CN, 30% H_2O; [d] 5% CH_3CN, 95% H_2O; [1] Poragel PT; [2] Phenyl-Corasil; [3] Poragel PS; [4] Poragel PN; [5] Active Fraction; [6] Side Fraction.

The yield of the most potent preparation of PIF which showed symmetrical single HPLC peaks was estimated to be ca. <100 ng from approximately 80,000 fragments. This estimate of yield was based upon a comparison of UV absorption data of the preparation in comparison with the absorption of synthetic model peptides ranging from 2-10 amino acids. Such an estimation of weight and consideration of purity are necessarily approximations, but when considered in conjection with biological potency, it appears that such a potent preparation could be essentially pure. This preparation seems to be considerably more potent than any such entity previously described by us or others (28,29).

The assay data in Table 2 show significant inhibitions in the release of prolactin. The best preparation (Table 2) inhibited, in vitro, the release of prolactin at a level of ca. <5 ng/ml medium. The paucity of this potent preparation severely restricted the exploration of the inhibitory activity.

The data of Table 2 also show that side fractions (SF) to the active fractions (AF) had little or no PIF activity. In the interest of hormonal specificity, the data in Table 2 show that the active fractions did not stimulate or inhibit the luteinizing hormone (LH) or the follicle stimulating hormone (FSH) or the growth hormone (GH) in companion radioimmunoassays. Particularly, the best preparation, which could be essentially pure PIF,

Table 3. In Vitro Bioassay Data on Intermediate Fractions[a] of PIF Derived from 240,000 Fragments[b]

Purification Step	Dose	ng P_2	PRL/ml I_2
DEAE-Sephadex	700	145	4
Sephadex LH-20	250	200	12
Poragel PT	500	230	0
Phenyl-Corasil	150	236	51
Poragel PS	160	274	9
SP-Sephadex	100	254	4

[a]See reference (27).
[b]These data are representative of a large number of columns at each step.

showed no activity of another hypothalamic releasing or inhibiting hormone for LH, FSH, or GH.

The data in Table 3 on intermediate fractions from 240,000 fragments showed the presence of this same PIF-entity, and there was virtually a total suppression of the release of prolactin by the representative fractions and assays.

An actual yield of only about <100 ng of a preparation showing such a potent PIF activity from about 80,000 hypothalamic fragments (% recovery unknown) indicates that structural elucidation of this prolactin inhibiting factor may require the processing of up to one-half million fragments as was necessary by others for the final elucidation of TRH (30), LHRH (31), and SRIF (32).

The achievement of this preparation of PIF and its potent activity gives new credence to the concept that the control of prolactin can be mediated by a complex mechanism including the participation of both a peptide inhibiting hormone and a catecholamine.

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