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Purification of a Non-dopaminergic and Non-GABAergic Prolactin Release-Inhibiting Factor (PIF) in Sheep Stalk-Median Eminence (41991)

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Abstract. Prolactin release-inhibiting factor (PIF) extracted from 1200 sheep stalk-median eminences was purified by gel filtration on a Sephadex G-25 column (4.5 × 150 cm). PIF activity was determined by measuring the inhibition of prolactin release from dispersed anterior pituitary cells of adult male or estrogen-primed, ovariectomized rats. Using this system, PIF was detected in tube fractions 122–127 (volume = 20 ml/tube). These fractions also contained LHRH and somatostatin; however, these peptides had no prolactin-inhibiting activity in the quantities present. No dopamine or γ-aminobutyric acid (GABA) was detected in the active fractions by radioenzymatic assay and fluorophotoenzymatic assay, respectively. Furthermore, receptor blockers for dopamine or GABA did not interfere with the PIF activity. These findings indicate that the PIF activity cannot be attributed to either dopamine or GABA, both of which are known to inhibit prolactin release, and provide evidence for the presence of a non-dopaminergic and non-GABAergic PIF within the hypothalamus.

Since dopamine (DA) was first reported to inhibit prolactin (Prl) release *in vitro* (1–3) and *in vivo* (4), DA has been considered to be a hypothalamic prolactin release-inhibiting factor (PIF). This view is supported by the following findings. Prolactin release can be suppressed by infusion of DA into portal vessels (5). DA is present in high concentration in median eminence (6) and in hypophyseal portal veins (7, 8), and DA receptors have been demonstrated on anterior pituitary cells (9, 10). The concentration of DA in hypophyseal portal vessels has been reported to be sufficiently high to suppress prolactin release which suggests that DA is a physiologically effective PIF (11). It is still not clear whether or not DA is the sole hypothalamic PIF. In fact, considerable evidence supports the existence of non-dopaminergic PIFs. For example, Schally *et al.* (12) isolated GABA from porcine hypothalamic extracts and showed it to have PIF activity *in vivo* and *in vitro*. Greibrokk *et al.* (13) obtained evidence for a peptidergic PIF in porcine hypothalamic extracts, whereas Enjalbert *et al.* (14) reported that DA-free subcellular fractions of rat hypothalamic tissue exhibited PIF activity. In the present study we describe the purification of a non-dopaminergic and non-GABAergic PIF in sheep hypothalamic extracts following gel filtration on Sephadex G-25. The PIF activity eluted in the same position as we had previously found it when measuring the activity by *in vivo* bioassay (15).

Materials and Methods. *Preparation of extracts.* Twelve-hundred ovine stalk-median eminences were collected at a slaughter house in San Angelo, Texas. The fragments were rapidly frozen on dry ice and lyophilized on return to the laboratory. The lyophilized tissue was extracted with an excess of acetone–0.01 *N* HCl (80/20 v/v) to a uniform consistency with a polytron. The mixture was centrifuged at 16,000*g* for 30 min and the supernatant was decanted. The residue was reextracted two additional times. The three supernatants were pooled and the lipids were removed by repetitive extraction with petroleum ether (40–60 bp), and the organic phase was discarded. The aqueous phase was concentrated by rotary evaporation and was then lyophilized.

Gel filtration. The lyophilized extracts equivalent to the 1200 hypothalami were dissolved in 10 ml of 0.2 *N* acetic acid and centrifuged at 25,000*g* for 30 min to remove small amounts of insoluble material. The supernatant was applied to a Sephadex G-25 column (4.5 × 150 cm, V_0 = 1060 ml) and 20-ml fractions were collected. The separation

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pattern was monitored by determining the absorbance at 278 nm.

Aliquots of 150 μl from three consecutive fractions (50 μl/fraction) were lyophilized and reconstituted in 1.0 ml of culture medium, which was then evaluated for PIF activity using dispersed cells from adult male rat pituitaries.

Biological assay for PIF. PIF activity was measured *in vitro* using overnight cultured dispersed anterior pituitary cells of adult male or estrogen-primed (50 μg of estradiol benzoate, sc, 2 days prior to sacrifice) rats. The procedure for dispersion has been previously described (16). Following overnight culture of the dispersed cells, the medium was removed and replaced by fresh tissue culture medium 199 (20 m*M* Hepes, 0.1% BSA, 100 U penicillin, 100 U streptomycin, 2×10^{-5} *M* bacitracin, Sigma, St. Louis, Mo.), pH 7.2–7.3, to which the sample had been added. When incubations were carried out with DA or spiroperidol, ascorbic acid (60 μ*M*) was added to prevent oxidation. Cells were incubated for 2 hr and the media were collected after centrifugation, decanted, and stored at −20°C until radioimmunoassay.

Chemicals. The following drugs were incubated with the cells to determine their effect on Prl release or to block PIF activity attributable to DA or GABA. They were (1) DA (Sigma), (2) GABA (Sigma), (3) spiroperidol (Janssen Pharmaceutica), and (4) bicuculline (Sigma). The doses used are reported under Results.

Measurement of catecholamines and GABA. Catecholamines (DA, norepinephrine, and epinephrine) were measured by radioenzymatic methods as reported by Ben-Jonathan and Porter (17).

GABA was detected by a fluorophotoenzymatic method following the instructions provided by Sigma Chemical Company with slight modifications. The principle of this method is the measurement of NADPH produced when GABA is metabolized to succinic semialdehyde and then to succinate in the presence of α-ketoglutarate, GABA transaminase, and succinic semialdehyde dehydrogenase. NADPH was chemically oxidized to NADP and measured fluorometrically at 340 μm. In our hands, the minimal detectable dose of synthetic GABA in this assay was 3×10^{-8} *M*.

RIA. Prolactin and thyroid stimulating hormone (TSH) released into the incubation medium were measured by the NIADDK RIA kits for rat Prl and TSH and expressed in terms of the RP-1 reference preparation. LHRH and somatostatin (SRIF) were determined by radioimmunoassay using antiserum kindly supplied by Dr. M. Igarashi (Gunma University, Maebashi, Japan) and Dr. L. R. DePalatis (University of Texas Health Science Center at Dallas), respectively. Statistical significance of differences among groups was evaluated by analysis of variance followed by Student–Newman–Keuls multiple comparison test.

Results. *Location of PIF in fractions from the Sephadex column.* Following gel filtration significant inhibition of Prl release was observed between fractions 122 and 127 (Fig. 1). The maximum inhibition was in fractions 125–127 and the magnitude of inhibition was about 25%. Slight but significant inhibition of prolactin release also occurred with fraction 90. The elution profiles of immunoreactive (IR)-SRIF, IR-LHRH, and TSH-releasing activity (TRF) evaluated by the TSH release into the incubation medium are schematically illustrated in Fig. 1, and it is apparent that the regions containing relatively high concentrations of SRIF and LHRH overlapped the active zone, whereas the area containing TRF activity was separated from that with PIF activity. The regions which contained potassium also overlapped the inhibitory zone for Prl release, whereas those fractions which contained sodium emerged somewhat earlier than the zone which contained PIF activity.

Since the magnitude of the inhibition was relatively small in this first experiment with dispersed cells from male rats, the same size aliquots were tested using dispersed cells from estrogen-primed, ovariectomized rat pituitaries, in which basal release was previously shown to be higher than that from cells of normal males. In this experiment a significant inhibition of release was observed beginning with fractions 122–124 and increasing with 125–127 ($P < 0.05$ and $P < 0.005$, respectively) (Fig. 2). The maximum inhibition occurred again with fractions 125–127 and was approximately 40%. Since the inhibition of

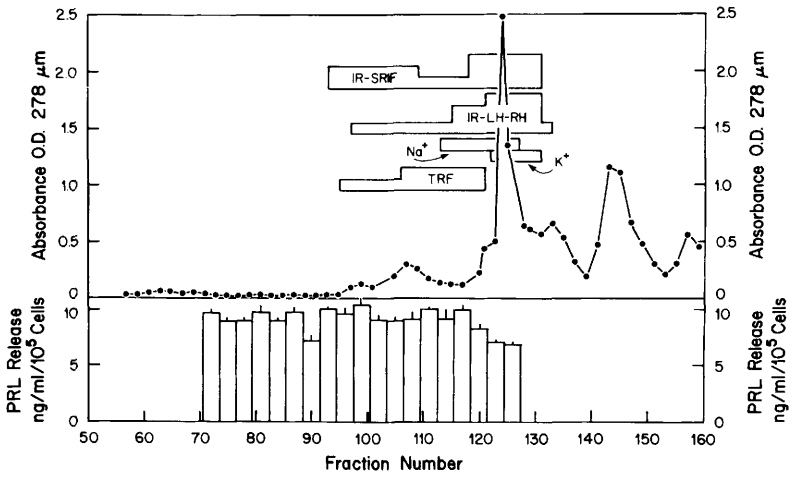

FIG. 1. Gel filtration of sheep hypothalamic extracts (1200 hypothalami) on Sephadex G-25 (column size 4.5 × 150 cm), solvent 0.2 *N* acetic acid, fraction size = 20 ml. Aliquots of 150 μl from three consecutive fractions (50 μl/fraction/ml incubation medium) were combined and their PIF activity was determined using a dispersed cell culture system of adult male rat pituitaries. *N* = 5 for each sample.

Prl release was more pronounced using cells from the estrogen-primed ovariectomized cells, the pituitary cells from this preparation were employed in the subsequent experiments.

Relationship of PIF activity to DA. To determine the relationship of the PIF activity to DA, several experiments were performed. The minimal effective dose of DA to decrease prolactin release significantly was 1×10^{-6} *M* and this effect was attenuated by coincubation with the same molarity of spiroperidol (*P* < 0.025) (Table I). On the other hand, the most active PIF fraction (fractions 125–127) induced a potent inhibition (50 μl of each fraction/ml of incubation medium) which was greater than that obtained with this dose of DA. The inhibition obtained with the PIF fractions was not altered by coincubation with spiroperidol. Spiroperidol alone at a dose of 5×10^{-7} *M* did not alter prolactin release from the cells; however, the higher dose (1×10^{-6} *M*), which significantly blocked the response to DA significantly inhibited Prl release when it was tested alone.

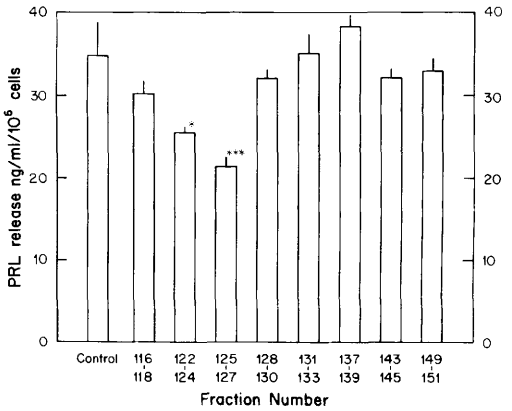

FIG. 2. PIF activity in aliquots of 150 μl from three consecutive fractions, determined by cell culture system of estrogen-primed ovariectomized rat pituitaries. **P* < 0.05, ****P* < 0.005 vs control, *N* = 4.

Relationship of PIF activity to GABA. To evaluate the possible role of GABA as a PIF, GABA was tested and had no effect on the release of Prl at a dose of 2×10^{-5} *M;* however, significant inhibition of Prl release was obtained by GABA at a dose of 8×10^{-5} *M* and the magnitude of this inhibition was not further altered by increasing the dose to 32×10^{-5} *M* (Table II). This result suggests that the inhibition had already reached a maximum at the dose of 8×10^{-5} *M*. Bicuculline, a GABA receptor blocker, on the other hand, induced a significant inhibition of Prl release at a dose of 2×10^{-5} *M* (*P* < 0.001). Consequently, the dose of bicuculline was reduced to 5×10^{-6} *M*. At this dose it did not alter release. This dose of bicuculline completely blocked the effect of GABA at a

TABLE I. EFFECT OF PIF FRACTION, SPIROPERIDOL, AND DOPAMINE ON PRL RELEASE *IN VITRO*

Treatment	*N*	PRL release (ng/ml/10^5 cells)	*P* vs control	*P* vs dopamine alone
Control	4	39.30 ± 3.03[a]		
Spiroperidol (5×10^{-7} *M*)	4	35.65 ± 3.75	NS	
Fraction 125–127 (50 μl/fraction/ml)	4	23.83 ± 3.02	<0.005	
Fraction 125–127 + spiroperidol (5×10^{-7} *M*)	4	23.35 ± 2.08	<0.005	NS
Dopamine (5×10^{-7} *M*)	4	29.56 ± 1.30	<0.10	
Dopamine (5×10^{-7} *M*) + spiroperidol (5×10^{-7} *M*)	4	35.40 ± 1.02	NS	<0.20
Control	5	61.03 ± 0.66		
Spiroperidol (1×10^{-6} *M*)	5	39.45 ± 2.36	<0.001	
Fraction 125–127 (50 μl/fraction/ml)	5	34.18 ± 2.72	<0.001	
Fraction 125–127 + spiroperidol (1×10^{-6} *M*)	5	34.40 ± 2.83	<0.001	NS
Dopamine (1×10^{-6} *M*)	5	41.33 ± 2.97	<0.001	
Dopamine (1×10^{-6} *M*) + spiroperidol (1×10^{-6} *M*)	5	50.55 ± 2.34	<0.005	<0.025

[a] Mean ± SEM.

dose of 8×10^{-5} *M* but failed to block the effect of the higher dose of GABA (32×10^{-5} *M*). The inhibitory activity of the PIF fractions, which was about 40%, was not altered by bicuculline.

Measurement of catecholamines and GABA in the fractions. To determine the concentration of catecholamines in the fractions, they were assayed for catecholamine content by radioenzymatic methods. The peak concentration of DA was eluted in fraction 139 (Table III); however, no dopamine was detected in the fractions which contained PIF.

To evaluate the role of GABA in the Prl release-inhibiting activity of the fractions, the GABA content of the extracts was measured (Fig. 3). As can be seen in the left panel, a dose-dependent increase of photoabsorbancy at 340 μm was observed in the presence of GABA at 2×10^{-8} to 32×10^{-8} *M*. The

TABLE II. EFFECT OF PIF FRACTION, GABA, AND BICUCULLINE ON PRL RELEASE *IN VITRO*

Treatment	*N*	PRL release (ng/ml/10^5 cells)	*P* vs control	*P* vs GABA alone
Control	7	56.78 ± 2.71		
Bicuculline (2×10^{-5} *M*)	5	37.58 ± 2.64	<0.001	
GABA (2×10^{-5} *M*)	5	55.73 ± 1.75	NS	
GABA (2×10^{-5} *M*) + bicuculline (2×10^{-5} *M*)	5	48.20 ± 1.10	<0.05	<0.05
Control	6	29.74 ± 1.58		
Bicuculline (5×10^{-6} *M*)	6	32.71 ± 1.96	NS	
Fraction 125–127 (50 μl/fraction/ml)	7	17.19 ± 1.03	<0.001	
Fraction 125–127 + bicuculline (5×10^{-6} *M*)	7	19.74 ± 0.86	<0.001	NS
GABA (2×10^{-5} *M*)	6	26.92 ± 0.92	NS	
GABA (8×10^{-5} *M*)	6	20.44 ± 0.74	<0.001	
GABA (8×10^{-5} *M*) + bicuculline (5×10^{-6} *M*)	6	29.14 ± 2.64	NS	<0.025
GABA (32×10^{-5} *M*)	6	20.51 ± 1.78	<0.001	
GABA (32×10^{-5} *M*) + bicuculline (5×10^{-6} *M*)	6	22.51 ± 1.78	<0.20	NS

TABLE III. CATECHOLAMINE CONTENT IN FRACTIONS GEL-FILTERED ON SEPHADEX G-25

Fraction	Dopamine	Norepinephrine	Epinephrine
125–127	ND	ND	ND
133	4.80 ± 0.00[a]	2.07 ± 0.02	1.32 ± 0.19
139	416.70 ± 6.30	186.80 ± 1.68	2.63 ± 0.16
144	17.10 ± 1.80	25.5 ± 3.18	0.14 ± 0.01
158	ND	0.06 ± 0.00	0.11 ± 0.07

Note. ND = not detected.
[a] Mean ± SE (ng/ml) (duplicate determinations).

absorbance was not altered by the PIF fractions, which indicates that they contained no detectable GABA.

Lack of effect of somatostatin on Prl release. Since there was an overlap of the fractions containing the PIF with those containing somatostatin, it was important to determine if somatostatin had any PIF activity. The somatostatin concentrations in the fractions containing PIF activity varied from 66 to 140 ng/ml. The profile of the somatostatin concentrations in the fractions is shown in Fig. 1. A range of dosages of somatostatin from 100 ng/ml to 1 μg/ml had no inhibitory effect on Prl release from the incubated cells (Table IV).

Discussion. The role of DA as a physiological PIF is generally accepted; however, DA levels in portal blood do not always reflect the magnitude of inhibition of Prl release (11, 18). Since portal blood is collected from anesthetized rats after extensive surgery, it is difficult to know whether or not these levels reflect the level present in portal blood of the normal rat. The conditions of collection may well produce dramatic alterations in the rate of release of DA into portal blood. It was shown earlier that the PIF activity in hypothalamic extracts was not abolished by DA receptor blocking drugs (19) and furthermore Enjalbert *et al.* (14) found PIF activity in catecholamine-free hypothalamic extracts. The present results clearly indicate the existence of a non-dopaminergic PIF in sheep hypothalamic extracts. Even the fraction (fraction 139) which contained the most DA (Table I) failed to inhibit prolactin release (Fig. 2), although this dose was greater than the dose of DA (1 μM) which produced an inhibition when added alone to the dispersed cells (Table I). We have no explanation for this discrepancy.

Our results further show that this PIF activity cannot be attributed to GABA, since our fractions contained no GABA and since

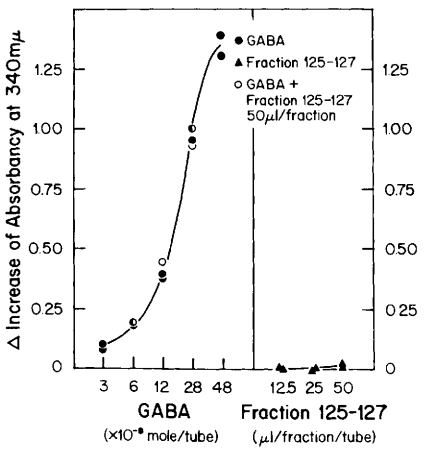

FIG. 3. Fluoroenzymatic determination of GABA in the PIF fractions (fractions 125–127). Aliquots of 150 μl from consecutive fractions (50 μl/each fraction) were lyophilized and reconstituted in 1.5 ml of buffer which contained GABA transaminase, NADP, and α-ketoglutarate.

TABLE IV. THE EFFECT OF VARIOUS DOSES OF SOMATOSTATIN ON PROLACTIN RELEASE FROM OVERNIGHT CULTURED CELLS OF OVARIECTOMIZED, ESTROGEN-PRIMED RAT

Treatment	*N*	PRL release (ng/ml)
Control	6	24.8 ± 3.0
SRIF		
100 ng/ml	5	29.5 ± 2.5
1000 ng/ml	4	23.9 ± 1.7

Note. Each flask had 275,000 cells. Values are means ± SEM.

the activity was not altered by the GABA receptor blocker, bicuculline, which antagonized the action of GABA. It is still possible that GABA plays a role as a PIF, since Schally *et al.* (12) isolated GABA from porcine hypothalamic extracts and demonstrated its PIF activity. Müller *et al.* (20) reported that larger relative amounts of GABA than DA were required to inhibit release *in vitro;* a result with which we concur. GABA may play a physiological role as a PIF since Prl was elevated in the conscious rat following the intravenous injection of the GABA receptor blocker, bicuculline (Mangat and McCann, unpublished data).

The fractions which we found to contain PIF activity overlapped the LH-releasing zone. Our results agree with the earlier report of Dhariwal *et al.* (15), who found an exactly similar pattern of PIF activity after gel filtration on an identical size column of Sephadex G-25. They further purified the PIF by chromatography on carboxymethyl cellulose but still were unable to separate the PIF from LHRH. Subsequently, Dular *et al.* (21) found PIF activity in fractions which also contained GH and TSH release-inhibiting activity but which were free of LHRH following gel filtration on Sephadex G-25. In our experiments the PIF zone also overlapped that containing IR-somatostatin. However, somatostatin in doses equal to or higher than those in the fractions with Prl-inhibiting activity had no effect on Prl release. GH release was significantly inhibited by the same fractions which contained PIF activity in our extracts which is indicative of their somatostatin content (data not shown). Under certain conditions, different from those employed here, somatostatin has been found to suppress Prl release (22, 23).

There were Na^+ and K^+ ions, particularly the latter, in the fractions which suppressed Prl release. It is unlikely that they could have been responsible for the inhibition of Prl release since these concentrations of Na^+ did not modify Prl release in the earlier fractions and since K^+ tends to elevate rather than lower pituitary hormone release (24). Furthermore, we have further purified this PIF by chromatography on carboxylmethyl cellulose which should render it essentially ion free (Mizunuma *et al.*, unpublished data).

It is tempting to speculate that the PIF in our fractions is peptidic in nature since it was eluted from the column in the position of other small peptides. Arguing in favor of this possibility, Khorram *et al.* (25) have recently found that pronase completely inactivates the PIF activity of crude hypothalamic extracts from infantile rats.

In conclusion these data clearly demonstrate the existence of a PIF other than DA and GABA in sheep stalk-median eminence extracts. It may be a peptide of approximately the same molecular weight as LHRH, but further experiments are necessary to prove this supposition. This PIF may be of considerable physiological importance.

We have not further investigated the apparent PIF activity found in tube 90. It is noteworthy that relatively high doses of the receptor blockers, spiroperidol and bicuculline, each lowered Prl release via the cells incubated *in vitro.* The ability of dopamine receptor blockers to lower Prl release has been observed earlier (19). This is the first report of the ability of bicuculline by itself to lower Prl release. The mechanism of the inhibitory action of these receptor blockers on Prl release is not understood but may be analogous to other situations in which receptor blockers have slight agonist activity. The lower dose of bicuculline did not alter Prl release but was effective in blocking the action of GABA. It did not alter the activity of the PIF-containing fractions.

In this paper elution positions have been expressed as the number of the fraction. Knowing the void volume of the column (1060 ml), it is possible to calculate the K_{av}. This was 1.09–1.12 for the PIF-containing fractions. The early elution position of TRH prior to the larger molecules, somatostatin-14 and LHRH, is peculiar, but has been reported repeatedly previously (26).

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