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Growth hormone releasing factor, somatocrinin, releases pituitary growth hormone *in vitro*

(hypothalamus/pancreas/tumor/somatostatin/perifusion)

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ABSTRACT **Purified (rat) hypothalamic growth hormone releasing factor (GRF), native human GRF isolated from an islet cell tumor of the pancreas that had caused acromegaly, and the synthetic replicates of the human material are potent secretagogues of immunoreactive growth hormone (GH) by primary cultures of rat pituitary cells. Native or synthetic peptides give identical dose–response curves, with identical slopes and identical maximal effects. The median effective dose of the tumor-derived GRF is 15 $\times 10^{-12}$ M. The effect of hypothalamic GRF or of a synthetic replicate of tumor-derived GRF is immediate, being demonstrable in ≤30 sec after contact in a pituitary cell perifusion system. The effect of hypothalamic GRF or of tumor-derived GRF is highly specific for stimulating release of immunoreactive growth hormone; there is no demonstrable concomitant effect on the secretion of other pituitary hormones. Somatostatin-28 and somatostatin-14 inhibit the release of growth hormone produced by hypothalamic GRF or tumor-derived GRF in typical noncompetitive antagonism. On the basis of the results reported here, hypothalamic GRF and tumor-derived GRF are qualitatively indistinguishable in their ability to stimulate the secretion of immunoreactive growth hormone *in vitro*. The name "somatocrinin" is proposed to replace the acronym GRF.**

We have characterized and reproduced by total synthesis a peptide with high intrinsic activity [median effective dose (ED_{50}) 15×10^{-12} M] in specific stimulation of secretion of immunoreactive growth hormone (GH) (1). This peptide [hpGRF-44, so designated to indicate its source (human pancreas), its action (growth hormone release), and its composition (number of amino acids)], along with two shorter fragments (hpGRF-37 and hpGRF-40) with reduced potency *in vitro*, were isolated from a human islet cell tumor that had caused acromegaly. From extracts of porcine or murine hypothalamus, on the other hand, we have obtained preparations of GRF of high purity and specific activity (ED_{50}, 50 pg/ml *in vitro*) but not in quantities sufficient for full characterization (2).

The data reported here show that the biological activities of purified hypothalamic GRF and homogeneous tumor-derived GRF, native or synthetic, are indistinguishable in several *in vitro* systems.

MATERIALS AND METHODS

Preparation of Cells for Monolayer Culture and Bioassay. Pituitaries from 30–40 male Sprague–Dawley rats (175 g) were removed aseptically after decapitation. The anterior lobes were collected, washed three times in sterile Hepes pH 7.35 buffer,* and dispersed at 37°C in 20–30 ml of Hepes buffer (pH 7.35) containing collagenase (Worthington CLSI-4197; 4 mg/ml) and dispase (protease grade II, Boehringer Mannheim II, 165-859; 2 mg/ml). After gentle vortexing and trituration by Pasteur pipette for 100–110 min, the dispersed cells were separated by centrifugation (150 × *g*, 4 min) and resuspended in Hepes buffer containing neuraminidase (Sigma N-2876; 8 μg/ml) and Na_2EDTA (Baker 1-8993; 0.2 mg/ml) at pH 7.35 for 10 min. The cells were washed twice with plating medium (defined below) and plated on multiwell plates (Falcon no. 3008; 1.5×10^5 cells per ml) or tissue culture plates (Falcon 3003; 4.5×10^6 cells per 25 ml) in the following defined medium: F-12/Dulbecco modified Eagle medium/BGjb, 6:3:1 (vol/vol) (GIBCO, 430-1700/430-1600/320-2591) with 2 g of bovine serum albumin, 2.38 g of Hepes, and 50 mg of garamycin (Schering) per liter plus 100 μg of cortisol, 1 μg of insulin, 0.4 μg of triiodothyronine, 0.2 μg of parathyroid hormone, 10 ng of glucagon, 0.1 μg of epidermal growth factor, 0.2 μg of fibroblast growth factor, and 10 mg of transferrin per liter. Plating was done with this medium containing 2% fetal calf serum to ensure rapid fixation of the cells. On the fourth day, the cells were washed twice with the defined medium without fetal calf serum. Finally, 900 μl of defined medium was added to each well with 100 μl of the same medium containing each individual treatment, in triplicate. After 3 hr of incubation the medium was collected and diluted as required for each radioimmunoassay (RIA).

Cell Preparation for Perifusion. On day 4 of culture as above in Falcon 3003 plates (25 ml, 2.5–3.0 pituitary equivalents), the cells were washed in defined medium and dispersed with a 1:250 trypsin/EDTA solution (GIBCO 610-5300) for 6–9 min. The dispersed cells were then deposited aseptically on a sterile Acrodisc filter (Gelman, no. 4192, 0.2 μm) and perifused with the defined medium for 2–3 hr before treatments were begun. The microchamber, as well as all solutions, was kept at 37–38°C in a small water bath with a needle bubble gas-feeder (5% CO_2/95% O_2). An adjustable peristaltic pump (Gilson Minipuls-2) was used to control the flow of the solution (≈500 μl/min) and a mini-fraction collector (Gilson) was used for collecting the perifusates.

Radioimmunoassays. RIAs for rat GH were performed with Sinha's anti-murine GH antiserum (3). RIAs for prolactin, thyrotropin, luteinizing hormone, and follicle-stimulating hormone were performed with the antisera provided by the Na-

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Abbreviations: RIA, radioimmunoassay; E_{max}, maximal effect of an agonist; ED_{50}, the dose of an agonist that produces 50% E_{max}; GH, immunoreactive growth hormone (somatotropin); GRF, growth hormone releasing factor.

* Hepes buffer contains per liter: 2.5 g of bovine serum albumin (Reheis, 2293-01), 10 mg of DNase (Sigma DN-100), 8 g of NaCl, 370 mg of KCl, 100 mg of Na_2HPO_4, 6 g of Hepes (Calbiochem 391,338), and 2 g of glucose. After dissolution and adjustment of pH to 7.30, this buffer is sterilized by passage through a 0.20-μm-pore filter (Millipore, Sybron 540-0020).

tional Pituitary Agency (A. Parlow, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases). RIAs for β-endorphin used antiserum RB-100 (4) prepared in this laboratory. Standard and experimental curves were calculated with the computer program described in ref. 5.

Peptides. *1. Hypothalamic GRF.* This was a purified preparation of GRF from rat hypothalamic extract. After gel filtration on Sephadex G-75 in 30% acetic acid, a step that removes all somatostatin-14 and most of somatostatin-28, the effluent with GRF activity was further purified by two steps of HPLC. The GRF preparation so obtained from 2,400 rat hypothalamic fragments was distributed into 1.0-ml vials in tissue culture medium and kept frozen at −20°C. Fifty microliters of this solution corresponds to the ED_{50} in a complete dose–response curve; that amount of the extract is defined as 1 unit of GRF activity, and that preparation of hypothalamic GRF is referred to as GRF reference standard (2).

2. Tumor derived GRF. Three peptides were isolated from a human islet cell tumor that had caused acromegaly, on the basis of their activity to stimulate secretion of GH *in vitro* (1). They are referred to as hpGRF-44, hpGRF-40, and hpGRF-37. Aliquots of native hpGRF-44 and hpGRF-40 as homogeneous materials were used in some of the experiments reported here.

3. Synthetic hpGRFs. All synthetic replicates of the tumor-derived GRFs were prepared by solid-phase synthesis methods as routinely used in this laboratory (6). When we refer to synthetic hpGRF-44 we imply that the molecule is in the amidated form, as is the native material (1); on the other hand, synthetic hpGRF-40 and hpGRF-37 refer to peptides in the free acid form, as are the native extracted peptides (1).

4. Somatostatin. Somatostatin-28 and -14 were synthesized by solid-phase methods (6).

Statistical Analyses. Comparison of the effects of various treatments to control values was done by the multiple comparison test of Dunnett following an analysis of variance (program EXBIOL) (7). Multiple dose–response curves in the bioassays were analyzed for simultaneous fitting by the four-parameter logistic equation of De Lean *et al.* (8) (program ALLFIT). The same data were also studied by regression analysis and calculations of relative potencies with 95% confidence limits (program BIOPROG) (9).

RESULTS

Specificity of GRF for the Release of Immunoreactive GH. When tested in the assay described above, purified hypothalamic GRF, native tumor-derived GRF-40, or synthetic hpGRF-44, at doses ranging from 0.6 to 40 units or 3 to 400 femtomoles per ml, respectively—which are known to reach maximal effect (E_{max}) for stimulation of GH secretion (Fig. 1)—released only immunoreactive GH; i.e., they had no effect on the secretion of immunoreactive β-endorphin, follicle-stimulating hormone, luteinizing hormone, thyrotropin, or prolactin (Table 1).

Dose–Response Relationships. In a large number of experiments, we have observed that purified rat hypothalamic GRF and native tumor-derived GRF-37, GRF-40, and GRF-44 as well as synthetic hpGRF-37, hpGRF-40, and hpGRF-44 all show identical dose–response curves when studied at doses ranging from 0.6 to 40 units of GRF reference standard for the hypothalamic material and 3–400 fmol/ml for the isolated or synthetic several hpGRFs. These doses are known to extend to E_{max} (Fig. 1). Calculations of the statistical fit of these curves by the four-parameter logistic polynomial model (8) confirmed that they have identical slopes (parameter *b*) and identical values for parameter *d* which represents the value of the E_{max} of an agonist. In other words, in the *in vitro* assay, purified hypothalamic GRF and the tumor-derived GRFs, native or synthetic, had identical effects and intrinsic activities: i.e., they activated the cell machinery involved in the release of GH through the same mechanism (action) and to the same maximal extent (effect).

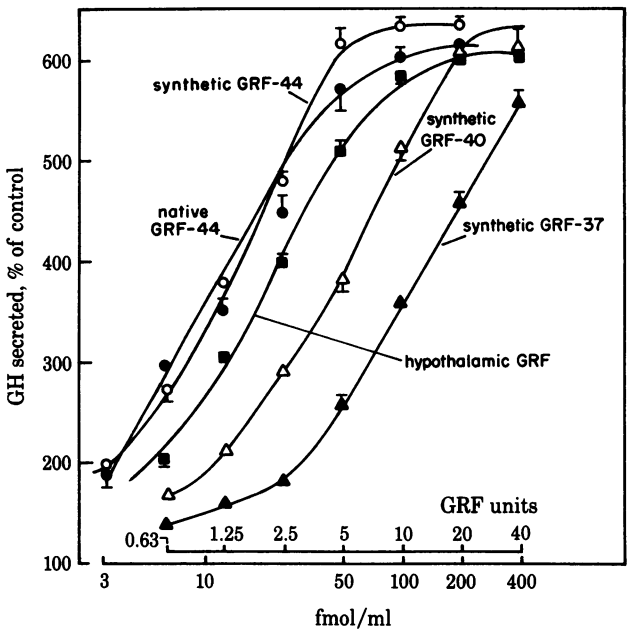

FIG. 1. Dose–response curves for multiple doses of hypothalamic GRF, native and synthetic hpGRF-44, synthetic hpGRF-40, and synthetic hpGRF-37. The vertical bar on symbols represents SEM; when no such bar appears, SEM is no greater than the height of the symbol. Lines are hand-drawn best fits (not theoretical regression curves).

The results show also that each of the three forms of tumor-derived GRF had a different specific activity. If the potency of native hpGRF-44 or of its synthetic replicate is taken as 100, in the assay described in Fig. 1 the potency of native or synthetic hpGRF-40 is 30 (95% confidence limits, 25 and 37) and that of native or synthetic hpGRF-37 is 12 (95% confidence limits, 9 and 16). The potency of native or synthetic hpGRF-44 is consistently greater than that of the other two forms isolated, with no overlap of confidence limits. From the calculated mean potency in six independent experiments, hpGRF-44 is 2.6 times more potent than GRF-40 (95% confidence limits, 2.3 and 3.2). From these results one can calculate that 1 unit of GRF activity in the purified hypothalamic extract used as the reference standard corresponds to ≈10 fmols of hpGRF-44. Thus, the extract from one rat hypothalamic fragment contains 350–500 fmol of GRF-44.

How Rapid Is the Effect of GRF in Eliciting Release of GH, and Is It Dependent on the Synthesis of Some Protein, Including GH? Results presented in Fig. 2 from one perifusion experiment with dispersed pituitary cells show that the stimulation of release of GH by hypothalamic GRF or synthetic hpGRF-44 was demonstrable in ≈30 sec after following contact of GRF with the pituitary cells. The effect of GRF was relatively short-lived, the duration of effect being related to the dose of GRF for identical pulse durations. The acute effect of synthetic hpGRF-40 on the release of GH was not modified by doses of cycloheximide as high as 100 μg/ml, added 2 hr prior to GRF (data not shown). These doses are well above those necessary to inhibit protein synthesis in the same *in vitro* system (10).

Antagonism Between GRF and Somatostatin. Somatostatin-28 and somatostatin-14 inhibited the response to hypothalamic GRF or native hpGRF-44 in a typical noncompetitive relationship (Fig. 3). Analysis of the dose–response curves by the four-

Table 1. Specificity of hypothalamic GRF and native or synthetic tumor-derived GRF to release GH but not other hormones

GRF tested	GH released, ng/ml	TSH released, ng/ml	PRL released, ng/ml	FSH released, ng/ml	LH release, ng/ml	βE released, pg/ml
Hypothalamic mGRF,* GRF units/ml						
0	870 ± 26	52 ± 3	384 ± 18	330 ± 19	1,056 ± 102	
0.63	1,710 ± 60	47 ± 2	347 ± 12	291 ± 21	901 ± 42	
1.25	2,626 ± 24	78 ± 19	394 ± 7	315 ± 12	1,012 ± 63	
2.50	3,923 ± 40	61 ± 11	375 ± 11	355 ± 6	1,077 ± 15	
5	5,586 ± 52	27 ± 7	410 ± 11	276 ± 16	820 ± 90	
10	6,803 ± 46	40 ± 7	386 ± 20	251 ± 17	873 ± 57	
20	7,060 ± 75	61 ± 6	424 ± 33	283 ± 7	856 ± 52	
40	7,213 ± 122	63 ± 17	475 ± 10	358 ± 25	1,169 ± 173	
Native tumor-derived hpGRF-40, fmol/ml						
6.3	1,903 ± 43	47 ± 6	485 ± 13	235 ± 5	761 ± 71	
12.5	2,163 ± 62	52 ± 13	371 ± 13	221 ± 20	679 ± 76	
25	3,480 ± 35	57 ± 6	388 ± 14	269 ± 11	901 ± 49	
50	4,820 ± 57	21 ± 4	406 ± 34	285 ± 6	947 ± 56	
100	6,746 ± 122	51 ± 4	369 ± 22	252 ± 24	827 ± 26	
200	7,070 ± 124	50 ± 3	432 ± 20	259 ± 23	844 ± 75	
400	7,606 ± 163	39 ± 1	405 ± 10	258 ± 4	856 ± 53	
Synthetic hpGRF-44, fmol/ml						
0	343 ± 12	308 ± 80	217 ± 12	125 ± 5	569 ± 35	1,157 ± 159
3.1	647 ± 9	416 ± 19	258 ± 18	130 ± 19	598 ± 42	1,153 ± 66
6.3	733 ± 13	382 ± 93	248 ± 3	142 ± 14	507 ± 34	1,054 ± 66
12.5	1,123 ± 12	297 ± 19	305 ± 42	170 ± 15	745 ± 25	1,151 ± 47
25	1,447 ± 7	304 ± 65	273 ± 17	140 ± 9	592 ± 47	997 ± 80
50	1,720 ± 30	343 ± 26	284 ± 9	179 ± 34	629 ± 34	1,001 ± 10
100	2,046 ± 17	307 ± 64	302 ± 10	181 ± 5	686 ± 37	1,225 ± 36
200	2,133 ± 13	377 ± 48	309 ± 5	146 ± 11	625 ± 10	1,226 ± 37

Results of two independent experiments are shown. In all cases, mean ± SEM shown come from duplicate RIA measurements for each treatment in triplicate—i.e., added to three tissue culture wells in the bioassay. Analysis of variance (EXBIOL) of all results for GH showed a highly significant treatment effect; subsequent linear regression analysis (BIOPROG) showed the results to be linearly distributed when effects and doses were related (see data in Fig. 1 for more evidence on this statement). For the other hormones, analysis of variance (EXBIOL) of all results showed no significant treatment effects. TSH, thyrotropin; PRL, prolactin; FSH, follicle-stimulating hormone; LH, luteinizing hormone; βE, β-endorphin.
* GRF reference standard.

polynomial equation (8) showed that values for parameter *b* (slopes) are statistically identical (for each set of curves), and so are values for parameter *c* (ED_{50}) for each set of curves. Similarity of the ED_{50} of the agonist (GRF) in the presence or absence of the antagonist (somatostatin) is one of the main criteria for noncompetitive inhibition. Values for parameter *a* (response at dose 0 of the agonist) and *d* (E_{max}) are different for each set of curves; dissimilarity of the values for *a* indicates that somatostatin also affects the basal secretion of GH by the pituitary cells *in vitro*. Dissimilarity of the values for *d* is another criterion of noncompetitive antagonism: the antagonist (somatostatin) acts at some locus other than the receptor of GRF to prevent the full activity of the agonist. The greater inhibition by somatostatin-28 than by equimolar amounts of somatostatin-14 (Fig. 3*b*) reflects the greater potency of somatostatin-28 when compared to the tetradecapeptide (11).

DISCUSSION AND CONCLUSIONS

The data presented here show that the biological activity of purified hypothalamic GRF is qualitatively undistinguishable from that of any of the three characterized forms of tumor-derived GRF or of their synthetic replicates. The slopes of the dose–response curves are identical and so are the values for E_{max} obtained with the hypothalamic or the tumor-derived preparations of GRF, native or synthetic. What differ are the specific activities (number of biological units per mole) of these various preparations. Native hpGRF-44 is the most potent of the three forms of hpGRF characterized. The potencies calculated for the native peptides are identical to those obtained for their synthetic replicates.

All evidence, however, points toward hpGRF-44 as the primary form of GRF. It is statistically more potent than any other form. It exists as a COOH-terminal amide (1), the form in which many neuropeptides [thyrotropin-releasing factor (TRF), gonadotropin-releasing factor (LRF), corticotropin-releasing factor (CRF), bombesin, substance P, vasopressin, oxytocin, etc.] and peptide hormones (α-melanocyte-stimulating hormone, secretin, cholecystokinin, gastrin, etc.) have been characterized and have maximal activity or are exclusively active. Moreover, the amino acid sequence of hpGRF-44 shows that hpGRF-37 and hpGRF-40 could be generated from hpGRF-44, by cleavage at the NH_2-terminal side of the arginine residues in positions 38 and 41, as has been found to be the case for dynorphin-8 (12) from dynorphin and for dynorphin B (rimorphin) from the COOH terminus of the β-neo-endorphin precursor (13).

Additional evidence for GRF-44 being the primary species and probably the form circulating in the hypothalamo–hypophysial portal system is the fact that several monoclonal antibodies raised against purified hypothalamic GRF (14) inhibit the activity of endogenous hypothalamic GRF but do not interfere with the biological activity of hpGRF-40 whereas in several ex-

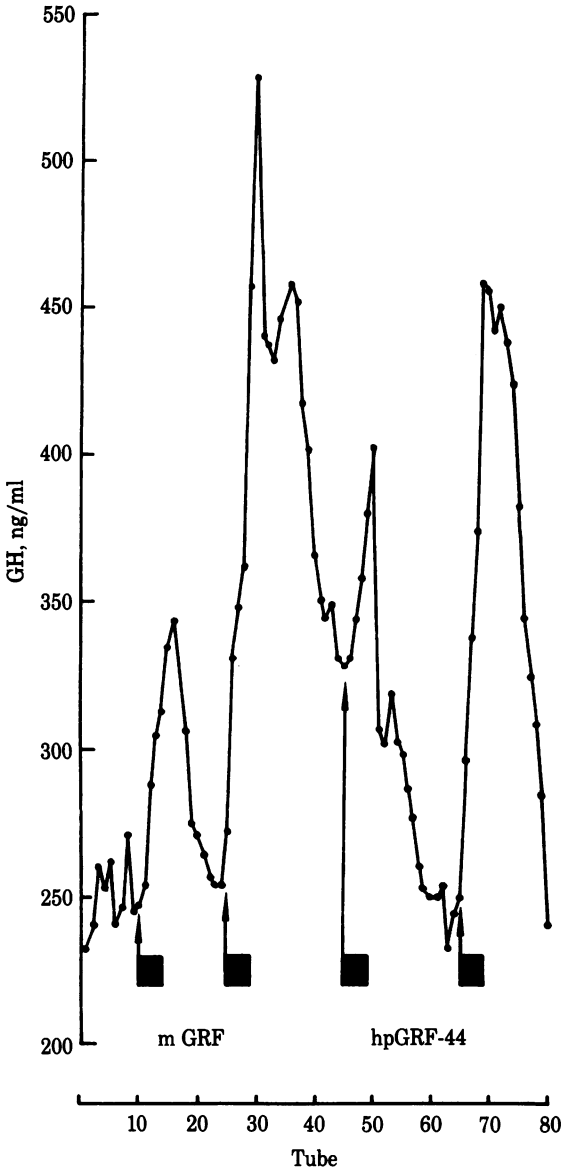

FIG. 2. Rapidity of the pituitary response to hypothalamic m (murine) GRF or synthetic hpGRF-44 in a perifusion system using dispersed pituitary cells. Each fraction collected is 250 μl and represents 33.8 sec; total duration of each GRF pulse was 155 sec. Doses: m GRF, 2 and 20 units (first and second squares); hpGRF-44, 20 and 200 fmol.

periments they bind hpGRF-44. Unpublished results in this laboratory show hypothalamic GRF activity to elute ahead of corticotropin-releasing factor activity in gel permeation experiments. More recently we have found that hypothalamic GRF activity elutes as synthetic hpGRF-44 in ion exchange HPLC.

The high specific activity (number of biological units per mole) of hpGRF-44 is worthy of comment. hpGRF-44 is significantly active in releasing GH in the monolayer culture assay at ≤3 fmol/ml or 3×10^{-12} M; in the dispersed pituitary cell perifusion assay the minimal active dose is ≤5 fmol/250 μl per 30 sec. The potency of hpGRF-44 thus is not only in the same range as that of the releasing factors for thyrotropin, the gonadotropins, or corticotropin but is even greater in comparable assay systems *in vitro*. Such high potency of the material along with its specificity for influencing the secretion of only GH favors physiological significance as a GH-releasing factor for hpGRF-44.

The noncompetitive nature of the inhibition of the activity of hypothalamic GRF or hpGRF-44 by somatostatin, although never reported as such by others (15–17), was not an unexpected finding. There is no evidence that somatostatin or any of its many analogs behave as a partial agonist (on the release of growth hormone); indeed, the latest proposal (18) for the subcellular mechanism of action of somatostatin would lead one to expect results consistent with a noncompetitive mode for the antagonism between somatostatin and GRF. These results are in agreement with earlier reports from this (19) and other laboratories (20) proposing that somatostatin-14 is a noncompetitive inhibitor of thyrotropin-releasing factor's effect on the secretion of thyrotropin and prolactin.

The rapidity of action of GRF, whether of hypothalamic origin or as hpGRF-44, is in keeping with the characteristics of other hypothalamic releasing factors for thyrotropin, gonadotropins, and corticotropin. Frohman *et al.* (16) used perifusion of whole rat pituitaries and found that the GRF material they had purified (from a human carcinoid that had caused acromegaly) also elicited rapid release of GH, although their experimental protocol was not as sharply designed as the one used here. In that (16) and a more recent report (21), Frohman *et al.* elaborated on various characteristics of the material with GRF activity that they had purified from several human tumors which had caused acromegaly. Many of their conclusions regarding molecular size, significance of the NH_2 and COOH termini, differences from the activity of hypothalamic extract, and conclusions as to existence of precursor forms of their active material are at variance with our present knowledge of the fully characterized hpGRF (1). It is always difficult to draw such conclusions when dealing with nonhomogeneous materials.

In keeping with requests of the nomenclature committees of the International Union of Physiological Sciences, the trivial name "somatocrinin" (1) is proposed for the growth hormone releasing factor to replace the acronyms GRF, GHRF, and GHRH.

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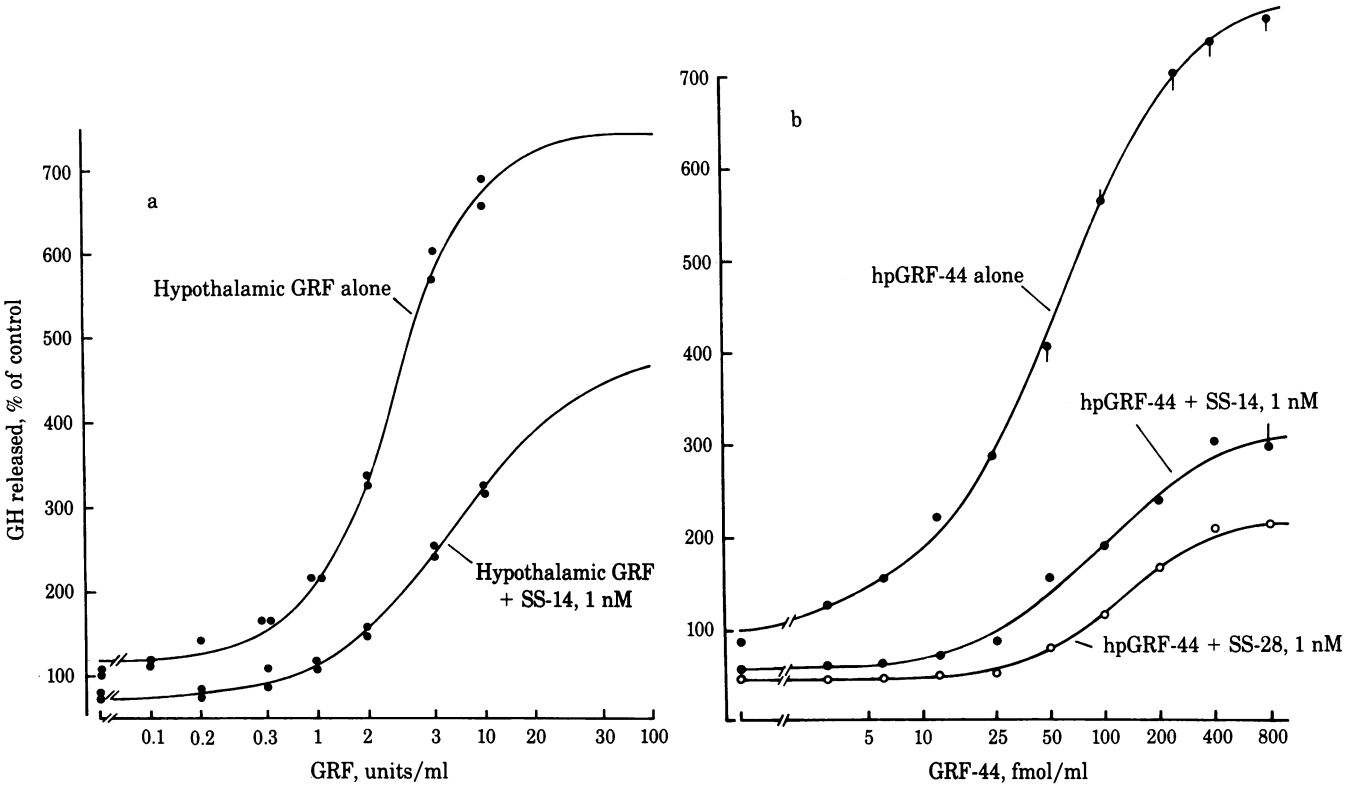

FIG. 3. Somatostatin-14 (SS-14) (*a*) or somatostatin-28 (SS-28) (*b*) inhibit the response to hypothalamic GRF or native hpGRF-44 in typical noncompetitive antagonism. Results of two independent experiments. Symbols show actual experimental data; lines are the theoretical curves computer-calculated and drawn from the four-parameter logistic equations for each set of data; curves shown here are drawn without constraints [program ALLFIT (8)].

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