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BINDING OF A GROWTH HORMONE RELEASING HEXAPEPTIDE TO SPECIFIC HYPOTHALAMIC AND PITUITARY BINDING SITES

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Summary—The drug SK&F 110679 (His-D-Trp-Ala-Trp-D-Phe-LysNH_2), is an enkephalin-derived hexapeptide, which specifically releases growth hormone in a wide variety of species *in vivo* and *in vitro*. Previous binding studies, using ligands which are specific for mu and delta opioid binding sites, demonstrated an inverse relationship between the opioid binding potency and the potency in releasing growth hormone of a series of peptides related to SK&F 110679. In an attempt to understand its mode of action better, a binding assay for the peptide was established using a ligand which had been tritium labelled at the D-Trp^2 residue. Membrane fragments from both the hypothalamus and anterior pituitary tissue were found to contain sites to which [^{3}H]SK&F 110679 reversibly and saturably bound. The binding curves for [^{3}H]SK&F 110679 to membrane fragments of both hypothalamus and anterior pituitary were resolved into two binding components with the computer program LIGAND. The K_d's obtained were in the 10^{-8} M and 10^{-5} M range. The relationship of these binding sites to the growth hormone-releasing activity of the peptide was explored by examining the relationship between the binding and potency in releasing growth hormone of a series of peptides related to SK&F 110679. For sites in both the hypothalamus and pituitary, a significant correlation between binding and the release of growth hormone was obtained. Thus, these binding sites appeared to be involved in the release of growth hormone by SK&F 110679-related peptides. Preliminary studies, designed to screen the ligands for previously established receptors, for their potential interaction with the binding of [^{3}H]SK&F 110679, have identified peptides which do inhibit binding. These peptides include growth hormone-releasing hormone of the rat, somatostatin, vasoactive intestinal peptide, dynorphin A and adrenocorticotrophic hormone (ACTH) (1–24). Detailed structure-activity studies may elucidate the relationship of these peptides to the curvilinear Scatchard graphs of the binding of [^{3}H]SK&F 110679 and to the role of the pituitary and the hypothalamus in the mode(s) of action of the hexapeptide.

Key words—growth hormone releasing peptide binding, growth hormone, SK&F 110679, growth hormone release.

Small, synthetic peptides have been used both to delineate structure–activity relationships in hypothalamic growth hormone-releasing factors and to investigate the regulatory mechanisms controlling the release of growth hormone. Analogs of enkephalin were modified at several positions to enhance their selectivity and potency for the release of growth hormone. Amidation of met-enkephalin gave it hypophysiotropic activity and substitution of D-Trp for glycine, at position 2, resulted in a peptide with releasing activity restricted to growth hormone (Bowers, Chang, Momany and Folkers, 1977). Subsequent structural modifications resulted in SK&F 110679 (His-D-Trp-Ala-Trp-D-Phe-LysNH_2; GH-RP-6) (Bowers, Momany, Reynolds and Hong, 1984) which not only is 1000-fold more potent in releasing growth hormone than is [D-Trp^2]-met-enkephalinamide, but also has growth hormone-releasing activity *in vivo* in a wide variety of species.

The precise structure–activity relationship observed among the growth hormone-releasing peptides, suggesting a receptor-mediated mode of action, led to the establishment of a binding assay for [^{3}H]SK&F 110679. Binding in the hypothalamus, as well as pituitary tissue, was investigated, since SK&F 110679 appears to act at both levels to modulate the release of growth hormone (Reynolds and Bowers, 1987).

METHODS

Animals and facilities

Male, Sprague–Dawley rats (3–4 months of age) were purchased from Charles River Breeding Laboratories (CD/VAF; Cambridge, Massachusetts) and acclimatized to local barrier conditions for at least 2 weeks before use. The rats were housed individually in wire hanging cages, under standard conditions of light (0600–1800 hr), temperature (22 ± 2°C), humidity (50 ± 10% relative) and nutrition (unlimited food and water).

Binding assay

The drug [^{3}H]SK&F 110679 was obtained by tritium-halogen replacement on the corresponding D-5,7-dibromotryptophan[2] peptide, which had been prepared by solid-phase peptide synthesis. The tritiated peptide was purified by reverse-phase high pressure liquid chromatography (HPLC). The specific activity, obtained from combined mass and radioactive determinations by high pressure liquid chromatography, was 29 Ci/mmol.

The rats were decapitated between 1000–1200 hr on the day of each experiment and their brains rapidly removed and dissected. The hypothalami and anterior pituitaries were placed in iced buffer (50 mM Tris-HCl, 2 mM ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA) pH 7.4). The tissues were homogenized in a Teflon-glass homogenizer, 1 ml of buffer per pituitary and 3 ml per hypothalamus. A 250 μl aliquot of these crude membrane suspensions was used for an assay tube and the final assay volume was 300 μl. For routine experiments, 1 mg/ml of bacitracin was added to the buffer solution used to dissolve peptides (and subsequently diluted to 0.1 mg/ml in the assay) to minimize adsorption of the peptides to the walls of the assay tubes. Incubation was carried out for 2 hr at 0°C and was terminated by filtration over Whatman GF/B filters which had been presoaked in 0.5% polyethylenimine. Tubes and filters were rinsed 3 times with 4 ml of buffer. Tissue radioactivity was measured by liquid scintillation spectrometry, using Protosol/Econofluor (New England Nuclear, Boston, Massachusetts).

Release of growth hormone

Data on *in vitro* release of growth hormone were taken from the work of Bowers *et al.* (Bowers, Momany, Reynolds, Chang, Hong and Chang, 1980; Momany, Bowers, Reynolds, Chang, Hong and Newlander, 1981; Momany, Bowers, Reynolds, Hong and Newlander, 1984). In these studies, the release of growth hormone from explanted pituitaries of rats, in response to stimulation by various peptides, was measured using a growth hormone radioimmunoassay for the rat. The relative potency of the peptides was calculated from dose–response data, assigning SK&F 110679 a value of 1000.

Analysis of data

Binding data for [^{3}H]SK&F 110679 were analyzed with LIGAND (Munson and Rodbard, 1980), a computerized nonlinear least squares program, weighted to account for the statistical distribution of the errors. The program was used to fit one or two binding-site models to the experimental results. Nonspecific binding was handled as a fitted parameter in the model and the computer run *F*-test was used to determine which model provided the best fit to the data.

Another binding analysis program, Lundon2, was used for analysis of the competitor ligands as this software includes calculation of Hill numbers from the binding data.

RESULTS

Because preliminary experiments indicated that the levels of radioactivity in solution were not stable over time, measurements were made of the tritium remaining in solution, in both glass and polystyrene test tubes, containing 15 nM [^{3}H]SK&F 110679 in buffers of various compositions. At the end of 3 hr in Tris buffer, only 35% of the radioactivity remained in solution in polystyrene test tubes and 65% in glass test tubes (Fig. 1). Inclusion of either (bovine serum albumin, BSA, 0.01%) or bacitracin (1.1 mg/ml) in the buffer used to dissolve the peptides prevented significant loss of the peptide from solution. Other substances tested (traysolol, leupeptin, bestatin, thiorphan, antipain, EGTA, NaCl) were ineffective in preventing tube-binding, but bacitracin was found to be effective at concentrations as small as 0.1 mg/ml. Thus, bacitracin was subsequently used in all buffers to which peptides were added; stock solutions of peptide contained 1 mg/ml bacitracin, making the final concentration in the assay about 0.1 mg/ml.

In initial experiments, [^{3}H]SK&F 110679 was found to bind to glass fiber filters during the usual filtration procedure. When the GF/B filters were presoaked in only Tris buffer, 38% of the total radioactivity was bound to the filters. Several substances were tested for their ability to minimize this binding. Polyethylenimine was not only the most effective agent in reducing binding to the filter but it, unlike some other substances tested, did not retard the rate of filtration, a process which would decrease the specific tissue binding being measured. Thus, a filter presoak of 0.5% polyethylenimine was used for all subsequent experiments.

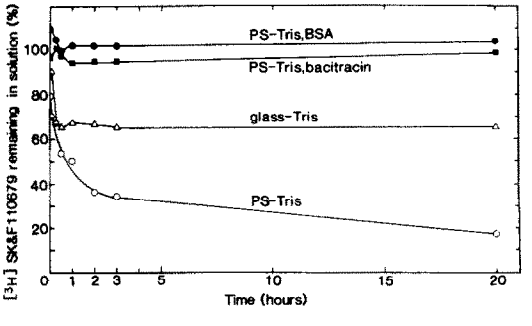

Fig. 1. Adsorption of [^{3}H]SK&F 110679 to test tubes under various conditions. The [^{3}H]SK&F 110679, at an initial concentration of 15 nM, was added to glass or polystyrene test tubes, containing Tris buffer only or Tris plus BSA (0.01%) or bacitracin (1.1 mg/ml). After an initial vortexing, aliquots were removed at various times and counted for radioactivity. The values shown are the means of triplicate determinations of radioactivity at each time.

Some experiments employed a crude mitochondrial fraction (P_2) (Burt and Snyder, 1975), prepared in phosphate buffer. Binding with this preparation was qualitatively similar to that obtained with Tris-EGTA homogenates, although some binding activity was lost during the centrifugation steps. Therefore the Tris-EGTA preparation was chosen, rather than the P_2. All experiments were conducted at concentrations of tissue at which binding was linearly dependent on the amount of tissue present.

Investigations of the ionic composition of the asay buffer included both monovalent and divalent cations. Reduced binding was observed in the presence of 5 mM $MgCl_2$, 100 mM NaCl, 5 mM $CaCl_2$ and 1 mM $MnCl_2$. Thus, no further additions were made to the Tris-EGTA buffer.

The binding of [^{3}H]SK&F 110679 to fragments of membrane from pituitary or hypothalamus reached equilibrium by two hours and declined slowly thereafter (Fig. 2). Thus, a 2 hr incubation at 0°C was used for further studies.

The reversibility of binding at 60 min was assessed by adding a large excess (1.67×10^{-5} M) of nonradiolabelled SK&F 110679 and measuring binding at subsequent times. The $T_{1/2}$s for the dissociation of [^{3}H]SK&F 110679 from pituitary and hypothalamus were 4 min and 1.5 min, respectively. Semilogarithmic plots of the dissociation data were not linear, a finding which supported the biphasic binding curves shown below.

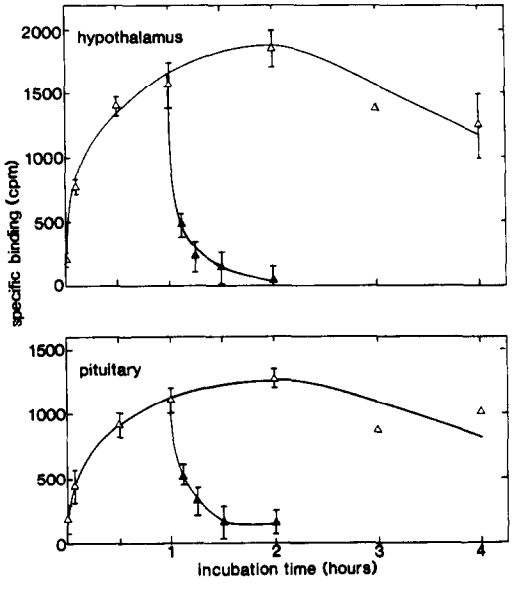

Fig. 2. Association (△) and dissociation (▲) time courses. The [^{3}H]SK&F 110679 (1 nM) was incubated on ice with a crude membrane preparation from the anterior pituitary and hypothalamus. At 1 hr, a large excess of nonradiolabelled SK&F 110679 was added to some tubes for the measurement of the dissociation time. The points indicated represent the means (±SD) of triplicate determinations of specific binding at the times indicated. Where error bars are not shown, they were smaller than the symbol.

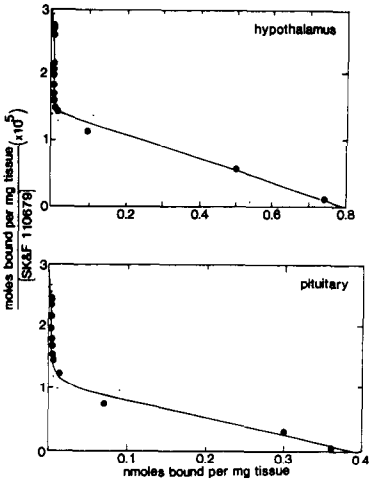

Fig. 3. Scatchard representation of the binding of [^{3}H]SK&F 110679 to pituitary and hypothalamic membrane fragments. Each data point shown represents the mean of triplicate determinations of specific binding of [^{3}H]SK&F 110679. The curves shown were fitted to the data with the nonlinear least-squares program LIGAND as described in Methods. For both tissue preparations, a two-site fit was a significantly better fit to the data than a one-site fit. The 95% confidence region around each curve is indicated by the small dots.

Scatchard graphs of binding data from both pituitary and hypothalamic tissue are shown in Fig. 3. The curves were fitted to the data with the computer program LIGAND (Munson and Rodbard, 1980). For both pituitary and hypothalamus, a significantly better fit to the data was obtained with a two-site fit than with a one-site fit ($P < 0.0005$, LIGAND-run F-test). The Hill coefficients, as expected when ligands exhibit curvilinear Scatchard plots, were less than 1 (0.36 for the pituitary and 0.28 for binding in the hypothalamus). The binding parameters, determined with LIGAND, are presented in Table 1. The K_ds were in the 10^{-8} and 10^{-5} M range and the densities of those binding sites were about 10^{-12} and 10^{-9} mol per mg of tissue. The nonspecific binding, when determined at 1 nM [^{3}H]SK&F 110679, with 100 μM SK&F 110679, was about 18% in pituitary and 37% in hypothalamic tissue.

The dose–response relationship of a series of peptides related to SK&F 110679 was investigated. These peptides exhibited a range of potencies in inhibiting the binding of [^{3}H]SK&F 110679; none of the Hill numbers differed significantly from 1. Figure 4 illustrates the linear relationship between the inhibition of

Table 1. Summary of binding parameters

Parameter	Pituitary[a]	Hypothalamus[a]
K_{d1}	72.0 ± 11.0 nM	20.7 ± 5.3 nM
K_{d2}	24.5 ± 3.1 μM	61.4 ± 7.3 μM
B_{max1}[b]	1.43 ± 0.31 pmol	0.10 ± 0.01 pmol
B_{max2}[b]	0.35 ± 0.11 nmol	1.07 ± 0.16 nmol

[a]Mean ± SEM.
[b]Mol per mg tissue.

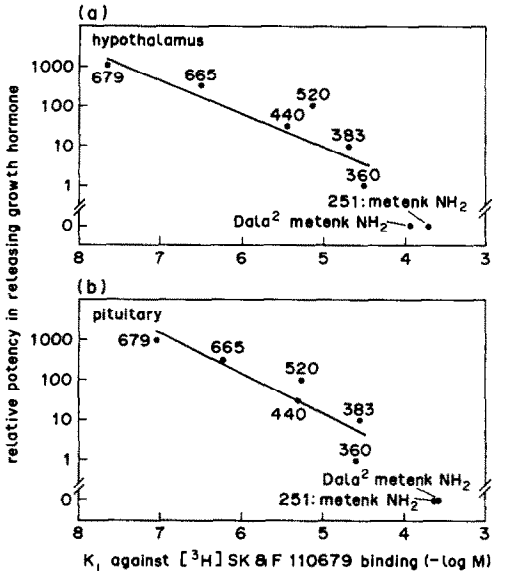

Fig. 4. Relationship between potency in releasing growth hormone and K_i for inhibition of the binding of [^{3}H]SK&F 110679. The K_i's for inhibition of the binding of [^{3}H]SK&F 110679 were determined from dose–response curves for each peptide in the presence of 1 nM [^{3}H]SK&F 110679. The growth hormone-releasing potencies were obtained from *in vitro* pituitary assays (Bowers *et al.*, 1980; Momany *et al.*, 1981; 1984). The lines were drawn by linear regression and the significance of the line determined with Correlation Analysis. For both pituitary and hypothalamic sites, a significant correlation between binding and the release of growth hormone was obtained. Amino acid sequences of the peptides are given in Table 2.

Table 2. Peptides used

Abbreviations	Identification
Peptides related to SK&F 110679	
251	Tyr-Gly-Gly-Phe-Met-NH_2
Dala^{2}metenkNH_2	Tyr-D-Ala-Gly-Phe-Met-NH_2
360	Tyr-D-Trp-Gly-Phe-Met-NH_2
383	Tyr-Ala-D-Trp-Phe-Met-NH_2
440	Tyr-D-Trp-D-Trp-Phe-Met-NH_2
520	Tyr-D-Trp-Ala-Trp-D-Phe-NH_2
665	His-D-Trp-Ala-Trp-D-Phe-NH_2
SK&F 110679, 679, GH-RP6	His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2
Other peptides	
ACTH (1–24)	adrenocorticotropic hormone
DAGO	Tyr-D-Ala-Gly-MePhe-Gly-ol
DPDPE	[D-Pen^{2,5}]enkephalin
rGHRH	growth hormone releasing hormone of rat
GRF antagonist	[*N*-Ac-Tyr^1,D-Arg^2]-GRF(1–29)NH_2
α-MSH	melanocyte stimulating hormone
rPHI	rat peptide histidine isoleucine
TRH	thyrotropin-releasing hormone
VIP	vasoactive intestinal peptide
VIP antagonist	[Ac-Tyr^1,D-Phe^2]-GRF(1–29)-NH_2

the binding of [^{3}H]SK&F 110679 by peptides in this series (see Table 2 for structures) and the growth hormone-releasing potencies of the peptides. For both the pituitary and the hypothalamus, the lines obtained by linear regression were significant fits to the data ($r = 0.90$ and 0.91; $P < 0.01$, Correlation Analysis), implying that the binding sites being measured in the present studies are involved in mediating SK&F 110679-induced release of growth hormone.

In a preliminary approach to the identification of the binding sites with which SK&F 110679 interacted, 1 nM of [^{3}H]SK&F 110679 was incubated with a large concentration (100 μM) of a number of potential inhibitory substances (see Table 2 for structures). The binding of [^{3}H]SK&F 110679 was inhibited not only by peptides related to SK&F 110679, but also by growth hormone-releasing hormone (rGHRH) of the rat, growth hormone-releasing factor (GRF) antagonist, vasoactive intestinal peptide (VIP), VIP antagonist, somatostatin, dynorphin A and adrenocorticotropic hormone (1–24) [ACTH(1–24)]. Peptides without significant inhibitory activity at this concentration included peptide histidine isoleucine of the rat (rPHI), galanin, DAGO (Tyr-D-Ala-Gly-NMe-Phe-Gly-ol), DPDPE ([D-Pen^{2,5}]-enkephalin), β-endorphin, β-casomorphin, α-melanocyte-stimulating hormone (α-MSH), thyrotropin-releasing hormone (TRH), bombesin and substance P (see Table 2).

Dose–response data were obtained with some of the peptides exhibiting inhibitory activity in the screening assays mentioned above. While the cost of peptides precluded investigation of full dose–response relationships, the computer models of the data obtained (Fig. 5) indicated that the peptides tested varied in potency, rather than in efficacy in inhibiting the binding of [^{3}H]SK&F 110679. Thus, the activity, observed in the single-dose screening assays, reflect differences in potency not efficacy.

DISCUSSION

Because most peptides have a net positive or negative charge at physiological pH, they have affinities for materials such as plastic and glass, making it necessary to take measures to ameliorate these effects. A wide variety of treatments has been used (to precoat the test tubes or filters, or in the incubation buffers themselves) to reduce these adsorption effects (Quirion and Gaudreau, 1985). In initial studies with the present system, artifactual binding of the ligand to test tubes reduced the effective concentration of [^{3}H]SK&F 110679 to only 35% of the level at the beginning (Fig. 1) and high levels of filter binding (38%) confounded measures of tissue-associated binding. However, preliminary experiments disclosed conditions under which binding artifacts were virtually eliminated, making possible the measurement of tissue-associated specific binding.

Using these protective agents, [^{3}H]SK&F 110679 was found to bind, saturably and reversibly (Fig. 2), to membrane fragments from both hypothalamus and anterior pituitary, the binding curves from both tissues being resolved into two binding components by computer analysis (Fig. 3).

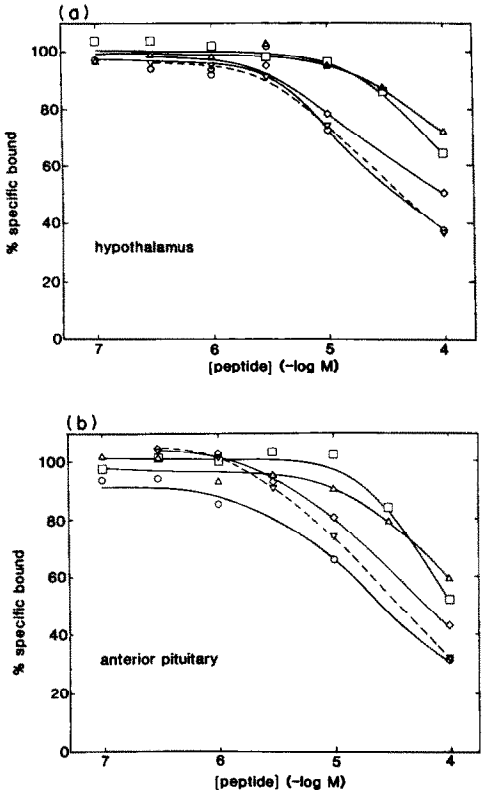

Fig. 5. Dose–response curves of the binding of [^{3}H]SK&F 110679 in the presence of some neuropeptides: GHRH (□), GRF antagonist (▽), somatostatin (△), VIP antagonist (◇), and dynorphin A (○). The concentration of [^{3}H]SK&F 110679 was about 1 nM. Specific binding was calculated with Lundon2; the curves shown represent single binding site models of the data.

The findings of binding sites for [^{3}H]SK&F 110679 in both the anterior pituitary and hypothalamus may relate to the proposed multiple sites of action of the peptide. *In vitro* assays have established that SK&F 110679 releases growth hormone (GH) directly from pituitary tissue (Bowers *et al.*, 1984; Reynolds and Bowers, 1987; Chao, Hoeffler and Frawley, 1988). But the activity of the peptide appears to involve an additional component at the level of the hypothalamus: *in vitro* release of growth hormone from pituitary and hypothalamic tissue incubated together, in response to stimulation by SK&F 110679, is greater than release from pituitary tissue alone (Reynolds and Bowers, 1987). Thus, SK&F 110679 may interact with receptors at both the pituitary and hypothalamic level to modulate the release of growth hormone.

Most importantly, inhibition of the binding of [^{3}H]SK&F 110679 by a series of enkephalin-derived, SK&F 110679-related peptides was directly related to the growth hormone-releasing potencies of the peptides (Fig. 4): K_is derived from the binding assay for [^{3}H]SK&F 110679 (herein described) correlate with the growth hormone-releasing potencies of the series of growth hormone-releasing penta- and hexapeptides, the structure-activity relationships of which have been extensively studied (Bowers *et al.*, 1977; 1980; 1984; Momany *et al.*, 1981; 1984; Codd, Yellin and Walker, 1988). This correlation strongly supports the concept that this binding site mediates the growth hormone-releasing action of SK&F 110679.

Because the present studies describe a binding site with properties that specifically relate to the growth hormone-releasing potency of SK&F 110679, they enabled investigation of what other substances might bind to these sites. Being derived from met-enkephalin, SK&F 110679 has the potential for interaction with opioid binding sites. However, previous studies have shown the interactions with both the mu and delta opioids of a series of SK&F 110679-related peptides to be inversely related to the growth hormone-releasing potency of the series (Codd *et al.*, 1988). That is, structural modifications which enhanced the growth hormone-releasing potencies of the peptides reduced their opioid binding potencies. Preliminary studies with [^{3}H]SK&F 110679 confirmed these findings: large concentrations of the mu and delta specific opioid ligands DAGO and DPDPE did not compete for the binding of [^{3}H]SK&F 110679 to either hypothalamic or pituitary membrane fragments.

Ligands for some other receptors involved in the regulation of the release of growth hormone did interact with the binding of [^{3}H]SK&F 110679: growth hormone releasing hormone of the rat, somatostatin and VIP significantly inhibited binding. Additionally, dynorphin A and ACTH (1–24) reduced the binding of [^{3}H]SKF 110679. The finding of multiple inhibitors of the binding of [^{3}H]SK&F 110679 may imply the interaction of SK&F 110679 with more than one receptor, a hypothesis supported by the curvilinear Scatchard plots of the binding of [^{3}H]SK&F 110679 (Fig. 3). Detailed structure-activity studies may help clarify not only the identity of the sites to which SK&F 110679 binds, but also the role of the hypothalamus as well as the pituitary in the modulation of the release of growth hormone by the hexapeptide.

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