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Binding of Nerve Growth Factor Receptor in Sympathetic Ganglia

(rabbit/superior cervical ganglia/peptide hormones)

SHAILESH P. BANERJEE, SOLOMON H. SNYDER*, PEDRO CUATRECASAS, AND LLOYD A. GREENE†

Departments of Pharmacology and Experimental Therapeutics, and Psychiatry and the Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; and † Laboratory of Biochemical Genetics, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT **^{125}I-Labeled nerve growth factor binds to membrane preparations from superior cervical ganglia of rabbit in a specific fashion indicative of an interaction with the physiological receptor. Of various tissues examined, binding was observed only in the superior cervical ganglia, a presumed target organ of nerve growth factor. Binding of labeled factor is displaced by nonradioactive factor but not by several other peptide hormones. Binding to ganglia of preparations of nerve growth factor treated with *N*-bromosuccinimide closely parallels their biological activity. Binding of nerve growth factor receptor is saturable with an affinity constant of about 0.2 nM, similar to the reported plasma levels of the factor. Nerve growth factor does not elicit insulin-like biological effects in isolated adipocytes, and it does not modify the binding of [^{125}I]insulin or [^{125}I]epidermal growth factor to fat cells or liver membranes.**

Nerve growth factor (NGF) is an important regulator of growth and development of sympathetic neurons (1). However, its exact mechanism of action is unclear and it is not certain whether its biological effects are restricted to the sympathetic and sensory nervous system. Selective accumulation of radioactive NGF by sympathetic ganglia (2) suggested that these ganglia might contain specific NGF-binding sites on their surface membranes. The similarities between the amino-acid sequences of NGF and proinsulin (3, 4), as well as similarities in the nature of some of the biological activities of these two hormones in their respective target cells (1, 3), suggested the possibility that the tissue receptors for NGF and insulin may possess some common features that could be investigated by procedures similar to those recently used to study insulin receptors (5–7). This report describes procedures for measuring the specific binding of NGF to membrane preparations from sympathetic ganglia (8) and presents the salient features of this NGF–receptor interaction.

METHODS

Fractionation of Rabbit Superior Cervical Ganglia. Sympathetic ganglia from young rabbits (Pel Freez, Birmingham, Ala.) and calves (Slaughterhouse, Baltimore, Md.) were homogenized in 10 volumes of 0.32 M sucrose containing 100 mM NaCl and 0.5 mM $MgSO_4$ with a Brinkmann Polytron Pt-10 (setting 2.2, 120 sec). The whole homogenate was centrifuged for 10 min at 1000 × *g*. The pellet (crude nuclear fraction) was suspended in 0.32 M sucrose containing 0.1 M NaCl and 0.5 mM $MgSO_4$, and the previous step was repeated. The two supernatant fractions were combined and centrifuged for 10 min at 7710 × *g*; the pellet (crude mitochondrial fraction) was suspended in Krebs–Tris buffer (pH 7.4) and the supernatant fluid was centrifuged for 60 min at 100,000 × *g* at 4°. The final pellet was suspended in Krebs–Tris buffer (2–5 mg of protein per ml) to obtain the crude microsomal fraction. This suspension was divided into multiple aliquots and stored either in liquid nitrogen or at −30° in a freezer. Stored in this way, binding of NGF receptor did not decline for at least 9 weeks. The protein content was measured (6). Rabbit ganglia were obtained freshly frozen and fractionated 3–10 days after the animals were killed. Calf ganglia were obtained fresh, frozen, and assayed 2 days later.

The Assay of Specific Binding of iodinated NGF to ganglia cell membranes was similar to that described for the binding of [^{125}I]insulin to cell membranes (6, 7). The usual binding assay consisted of incubation of ganglia cell membranes at 24° for 40 min in 0.25 ml of Krebs–Tris buffer (pH 7.4) containing 0.1% (w/v) crystalline bovine-serum albumin (Sigma Chemical Co.) and [^{125}I]NGF. After incubation, 3 ml of ice-cold Krebs–Tris buffer with 0.1% albumin was added to each tube and filtered and washed over EHWP (Millipore filters) (5–7). Corrections were made for nonspecific binding of iodo-NGF to the membrane and to the filters by running parallel incubations in which excess unlabeled NGF (10 μg/ml) was added to the membranes before [^{125}I]NGF. Specific binding was obtained by subtracting from the total radioactive uptake the amount that was not displaced by native NGF.

The 2.5S preparation of NGF, prepared by the method of Bocchini and Angeletti (9), was used for all the experiments in this study unless otherwise indicated and will henceforth be referred to simply as NGF, while other preparations will be specifically designated. The β-subunit of 7S NGF was prepared by the method of Varon *et al.* (10). The biological activity of NGF preparations was assayed by measurement of neurite production in dissociated sympathetic cells of chick embryo (L. Greene, in preparation). In this assay, 10 units correspond to 1 unit in conventional bioassays for NGF (11). This assay is more sensitive than the conventional bioassay and permits detection of changes in activity that vary by a factor of less than 2. In the conventional bioassay, one BU is referred to as the amount of NGF required to give a 4+ response in the tissue-culture system. The biological activity of NGF preparations used in this study varied from 30.0–40.0 units/μg of protein when the unit is defined as described by Levi-Montalcini *et al.* (11).

NGF was iodinated by procedures similar to those described for insulin (5–7) except that the sodium metabisulfite step was omitted. NGF (10–20 μg) was mixed with 1–3 mCi of freshly prepared, tracer-free Na^{125}I (Union Carbide) in 0.12 ml of 0.25 M sodium phosphate buffer (pH 7.4). Chloramine

Abbreviation: NGF, nerve growth factor.

* To whom reprint requests should be sent at the Department of Pharmacology.

TABLE 1. *Specific binding of [^{125}I]NGF to subcellular fractions of rabbit sympathetic ganglia*

Fraction	Specific binding (cpm/mg of protein)	Total binding per fraction (cpm × 10^{-5})
Whole homogenate	6880 ± 110	14
Crude nuclear fraction (1000 × *g*, 10 min)	4860 ± 40	2
Crude mitochondrial fraction (7710 × *g*, 10 min)	9040 ± 140	0.9
Crude microsomal fraction (100,000 × *g*, 10 min)	62,900 ± 330	11

Binding of 4 ng/ml of [^{125}I]NGF was assayed in a total volume of 0.25 ml. For each experimental point, nonspecific binding in the presence of 10 μg/ml of native NGF was subtracted. Data for specific binding are the means ± SEM of four samples.

T (20 μl of 0.5 mg/ml) was added, and after 20–30 sec at 24° 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) was added. The solution was rapidly added to and mixed with one 25-mg talc tablet (Gold Leaf Pharmacol) in a conical centrifuge tube. After 1 min, 9 ml of 0.1 M sodium phosphate buffer containing 0.1% albumin were added. The talc was washed five times with this buffer before the sample was eluted (5–7) with 1 ml of 0.5 N HCl–1% albumin. Different samples of purified [^{125}I]NGF, prepared over an 8-month period, contained 1 to 3 × 10^6 cpm/20 μl, were 85–97% precipitable by 7% trichloroacetic acid (5–7), and had specific activities that varied from 40–100 μCi/μg. The β-subunit was iodinated in the same way. [^{125}I]NGF preparations were stable for about 2 months when stored at 4°; nonspecific binding of [^{125}I]NGF to membranes generally decreased during such storage.

RESULTS

General Properties and Kinetics of Binding. [^{125}I]NGF binding is linearly dependent on the concentration of membrane protein of superior cervical ganglia over the range 1–10 μg and plateaus beyond 20 μg (Fig. 1). All assays were therefore conducted in the linear range.

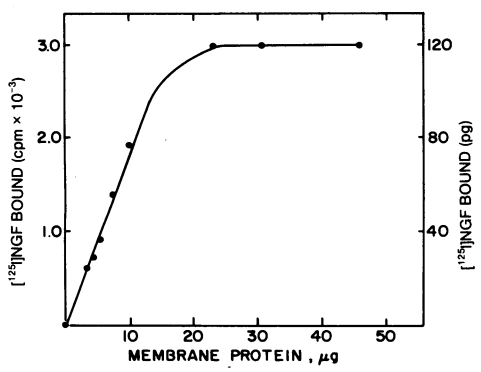

FIG. 1. Effect of membrane protein concentration on the specific binding of [^{125}I]NGF. Various amounts of microsomal protein obtained from rabbit sympathetic ganglia were incubated for 40 min at 24° with 6.5 ng/ml of [^{125}I]NGF (30,000 cpm) in a total volume of 0.25 ml. Each point is the mean of duplicate determinations.

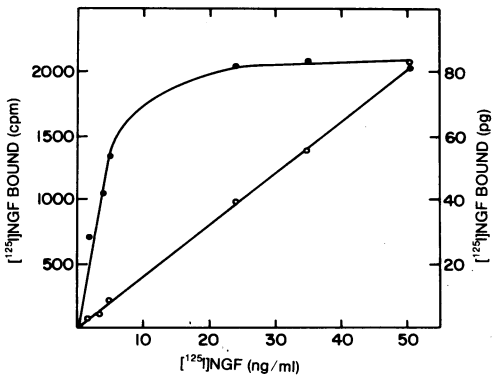

FIG. 2. Saturation of specific NGF binding to membranes from sympathetic ganglia. Binding was measured by incubation of various amounts of [^{125}I]NGF with microsomal preparations of rabbit sympathetic ganglia (8 μg of protein) in a total volume of 0.25 ml of Krebs buffer for 40 min at 24°. For every concentration of [^{125}I]NGF studied, control incubations were performed in the presence of 10 μg/ml of native NGF to obtain nonspecific binding. This nonspecific binding (○) has been subtracted from the total binding to obtain the specific binding curve (●).

The specific binding of [^{125}I]NGF is saturable with respect to the hormone (Fig. 2). Saturation occurs at about 20–30 ng/ml of [^{125}I]NGF, and half-maximal binding is observed at about 6 ng/ml (0.2 nM). By contrast, the binding of [^{125}I]-NGF in the presence of excess nonradioactive NGF ("nonspecific" binding) is not saturable and increases linearly with increasing amounts of [^{125}I]NGF. At concentrations of NGF lower than 5 ng/ml the ratio of specific to nonspecific binding is about 10. A maximum of 0.2 pmol of [^{125}I]NGF is bound per mg of membrane protein.

Competition for binding of [^{125}I]NGF by native NGF was examined by incubation of membrane preparations of ganglia with [^{125}I]NGF (6 ng/ml) that was mixed with different concentrations of native NGF (Fig. 3). The pattern of displacement observed is consistent with a similarity in the behavior of native and iodinated NGF with respect to binding to membrane receptors. The concentration required to achieve half-maximal displacement of binding is about 7 ng/ml of native NGF (0.2 nM). The fact that half-maximal saturation (Fig.

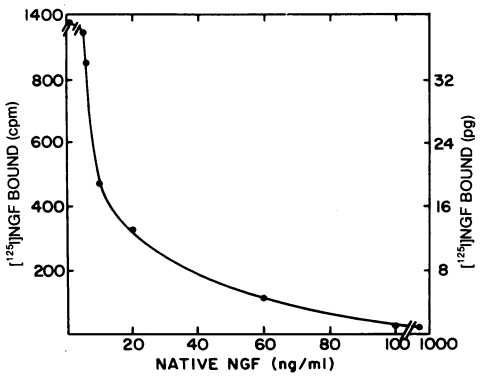

FIG. 3. Competition by native NGF of the specific binding of [^{125}I]NGF to microsomes obtained from rabbit ganglia. Microsomal suspensions (7 μg/ml of protein) were incubated with 6 ng/ml of [^{125}I]NGF (25,000 cpm) and increasing amounts of native NGF at 24° for 40 min. Nonspecific binding obtained in the presence of 10 μg/ml of native NGF has been subtracted from all experimental points.

2) occurs at identical concentrations of [^{125}I]NGF indicates that the [^{125}I]NGF is equivalent to native NGF in terms of receptor binding and confirms the validity of the determined specific activity of [^{125}I]NGF. When displacement experiments were repeated with ^{125}I-labeled β-subunit of 7S NGF and unlabeled β-subunit, the patterns of competition were indistinguishable from those obtained with NGF.

Specific binding occurs rapidly at 24°. A plateau is reached by 12 min and half-maximal values occur at about 1 min (Fig. 4). This information permits calculation (5) of the apparent bimolecular rate constant of NGF-receptor association α_1, which is 4×10^6 M^{-1} sec^{-1}. The rate of dissociation of the NGF–receptor complex can be measured directly at 24° and 4° (Fig. 4). At 24° dissociation is linear when plotted semilogarithmically; the half-life of the complex is about 13 min. The rate constant for dissociation at 24° is 9×10^{-4} sec^{-1}. Dissociation is very slow at 4°; less than 20% of bound [^{125}I]-NGF dissociates in 20 min.

Subcellular Localization. To determine the relative amounts of NGF-binding activity in different subcellular fractions, we homogenized ganglia and subjected them to differential centrifugation (Table 1). The highest specific activity of binding of [^{125}I]NGF occurs in the crude microsomal fraction, which is enriched in membrane fragments. The specific activity of binding in the crude microsomal fraction is almost 10-times greater than that of the whole homogenate and about 13-times greater than that of the crude nuclear fraction, which contains the lowest specific activity of NGF binding. Of the total binding activity in the whole homogenate, 79% is recovered in the microsomal fraction. Since almost all of the binding activity of the homogenate is recovered in the three subcellular fractions, it is probable that only a negligible portion of the total binding activity is present in the supernatant fraction, which could not be assayed directly.

Tissue Distribution. To determine whether NGF binding represents binding to the physiological NGF receptor, we evaluated several tissues in addition to superior cervical ganglia (Table 2). In these experiments superior cervical ganglia of rabbits bind more than 4000 cpm of [^{125}I]NGF, and the binding is reduced more than 10-fold by addition of excess nonradioactive NGF. Although binding of [^{125}I]NGF is observed with several other tissues, in all cases there is no significant reduction of binding in the presence of 10 μg/ml of nonradioactive NGF.

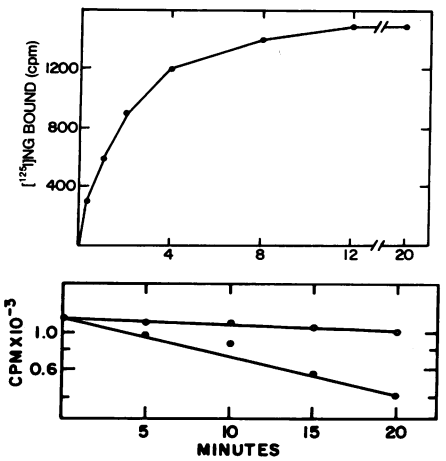

FIG. 4. Rates of association and dissociation of [^{125}I]NGF to microsomes obtained from rabbit sympathetic ganglia. *Upper:* The rate of binding at 24° of 4 ng/ml of [^{125}I]NGF to microsomes (8 μg/ml of protein). Nonspecific binding has been subtracted from all experimental points. *Lower:* Semilogarithmic plot of the dissociation of [^{125}I]NGF bound to microsomes as a function of time at 4° (○) and 24° (●). The procedure for determination of the rate of dissociation has been described (5).

TABLE 2. *Tissue specificity of [^{125}I]NGF binding*

Tissue	[^{125}I]NGF bound (cpm)	
	Without native NGF	10 μg/ml of native NGF
Rabbit superior cervical ganglia	4210	326
Calf superior cervical ganglia	3170	628
Mid-brain	701	804
Adrenal glands	713	893
Liver	1500	1390
Heart	500	505
Kidney	571	522
Fat cells	1390	1300

The indicated tissues, obtained from rabbits (except fat cells and calf ganglia), were homogenized in 10 volumes of 0.32 M sucrose with 100 mM Na^+ and 0.5 mM Mg^{++}. Isolated microsomes were suspended in the same volume of Krebs–Tris buffer, and 20 μl of this suspension was used in the standard binding assay. Incubation medium contained 6 ng/ml of [^{125}I]NGF in a total volume of 0.25 ml. Fat cells were obtained from rats and contained about 4×10^5 cells per ml (5). Data are the mean results from three determinations.

Specificity of Binding Interaction. To determine whether binding of NGF to superior cervical ganglia is specific for NGF, we examined the ability of various other peptide hor-

TABLE 3. *Effect of peptide hormones on binding of [^{125}I]NGF and [^{125}I]epidermal growth factor to preparations of rabbit sympathetic ganglia membrane*

Iodohormone	Hormone added	[^{125}I]-Hormone binding (cpm)
[^{125}I]NGF (4 ng/ml)	No addition	3370 ± 330
	NGF (5 μg/ml)	742 ± 70
	Glucagon (5 μg/ml)	3270 ± 180
	(50 μg/ml)	3160 ± 230
	(500 μg/ml)	3020 ± 210
	Growth hormone (10 μg/ml)	3240 ± 170
	(100 μg/ml)	3030 ± 260
	(1 mg/ml)	2510 ± 190
	Insulin (500 μg/ml)	3260 ± 280
	Proinsulin (500 μg/ml)	3010 ± 160
	Desoctapeptide insulin (1 mg/ml)	3260 ± 220
[^{125}I]Epidermal growth factor	No addition	262 ± 20
	NGF (100 μg/ml)	248 ± 20

Data presented are the mean of four determinations ± SEM.

TABLE 4. *Effect of NGF on the specific binding of [^{125}I]insulin and [^{125}I]epidermal growth factor to various tissues*

Tissue	[^{125}I]Hormone added	Native hormone		Binding (cpm)
		Hormone	Concentration (μg/ml)	
Fat cells	Insulin (1.2×10^5 cpm)	None		14,700
		Insulin	0.1	7,100
		Insulin	1	4,610
		NGF (7 S)	100	14,700
		NGF (7 S)	1	15,200
		NGF (β-subunit)	100	14,300
		NGF (β-subunit)	0.1	14,400
Liver membrane	Insulin (9.4×10^4 cpm)	None		5,780
		Insulin	0.1	940
		NGF (7 S)	100	5,860
		NGF (β-subunit)	100	5,610
	EGF (6.8×10^4 cpm)	None		28,300
		EGF	0.01	5,220
		NGF (7 S)	100	29,100
		NGF (β-subunit)	100	28,500

Fat cells (1.1×10^5 cells per ml) and liver membranes (210 μg of protein) were incubated at 24° for 30 min in 0.25 ml of Krebs–Ringer-bicarbonate buffer (pH 7.4) containing 0.1% albumin and the indicated concentrations of [^{125}I]insulin (140 Ci/g), [^{125}I]epidermal growth factor (*EGF*) (96 Ci/g), and the indicated native hormone. The latter was added to the membranes before addition of the iodohormone. Binding was determined as described (5–7); all values are corrected for nonspecific binding. Many similar experiments were performed with various concentrations of the iodoproteins with a broad range of concentrations of NGF; in no case did NGF cause significant displacement of binding.

mones to compete with [^{125}I]NGF for binding to ganglia (Table 3). The following peptides do not displace NGF binding in any of the concentrations examined: glucagon, insulin, proinsulin, and desoctapeptide insulin. Growth hormone at 1 mg/ml reduces binding by 25% but fails to affect NGF binding when used at 10 or 100 μg/ml.

The ability of NGF to interfere with the binding of [^{125}I]-epidermal growth factor to sympathetic ganglia was also examined. The limited amount of binding observed was not displaced by excess quantities of nonradioactive epidermal growth factor or NGF. Since epidermal growth factor and NGF are similar in their source (12), the apparent failure of epidermal growth factor to bind to the NGF receptor sites provides further evidence in support of the specificity of NGF binding.

TABLE 5. *NGF specific binding and biological activity after N-bromosuccinimide treatment*

N-Bromosuccinimide (nmol/μg of protein)	Biological activity (units/μg of protein)	Tryptophan % of untreated control	Percent displacement specific [^{125}I]NGF binding	
			NGF concentration (5.5 ng/ml)	(55.0 ng/ml)
None	333	100	52	91
0.30	108	45	—	55
0.43	49	18	17	27
0.57	0	0	8	4

The binding experiment was done as described in the *text* and native NGF was treated with *N*-bromosuccinimide by the procedure of Spande and Witkop (13). The specific binding of [^{125}I]NGF to ganglia microsomal preparations in the absence of NGF treated with *N*-bromosuccinimide was 3630 cpm.

Under various conditions it was not possible to demonstrate significant effects of native NGF on the specific binding of [^{125}I]insulin to intact fat cells or liver membranes (Table 4). NGF also does not modify the specific binding of [^{125}I]epidermal growth factor to liver membranes (Table 4).

As a further test of the physiological relevance of NGF binding, the biological activity of NGF was correlated with its ability to bind to membrane fragments of superior cervical ganglia of rabbits. NGF preparations were treated with various amounts of *N*-bromosuccinimide (13), which destroys the biological activity of NGF by selectively oxidizing tryptophan residues (14). Treated preparations of NGF with a range of biological activity were prepared (Table 5). The reduction in biological activity of the various preparations correlates well with the content of tryptophan. Bromosuccinimide-treated samples that are devoid of biological activity do not displace [^{125}I]NGF binding. A preparation containing 40% of the biological activity of the native hormone displaces [^{125}I]NGF binding half as well as native NGF, while a sample containing only 14% of control biological activity decreases binding 27% as well as native NGF. Treatment of [^{125}I]NGF with bromosuccinimide (0.6 nmol of bromosuccinimide per μg of protein) destroys its specific binding to ganglia membranes.

Absence of Inactivation during Binding Process. To determine whether NGF is degraded as a consequence of receptor binding, we studied the binding capacity of [^{125}I]NGF eluted from ganglia membranes. 2 mg of ganglia-membrane protein were incubated with 50 ng (2×10^6 cpm) of [^{125}I]NGF at 4° for 10 min in a total volume of 1 ml. After the preparation

was centrifuged at 50,000 × g for 10 min, the pellet was extracted with 1 ml of 0.1 N HCl at 37° for 30 min and centrifuged. The resultant solution was neutralized to pH 7.0 with 0.2 M sodium phosphate buffer (pH 7.9). This preparation was assayed for binding to ganglia membranes along with identical amounts of untreated [^{125}I]NGF. [^{125}I]NGF eluted in this way from plasma membranes binds to the same extent as comparable amounts of untreated [^{125}I]NGF. Binding of both preparations is similarly displaced by excess NGF (10 μg/ml). Thus, NGF resembles insulin (5) in remaining biologically intact after binding to receptor sites.

NGF and Insulin-Like Activity. Extensive efforts were made to determine if NGF could elicit insulin-like biological effects in isolated fat cells. Whereas insulin (2 ng/ml) caused a 7-fold enhancement in conversion [^{14}C]glucose (0.2 mM) to [^{14}C]CO_2 during a 2-hr incubation (15) at 37°, NGF in concentrations ranging from 1 ng/ml to 100 μg/ml was without effect. Various attempts were made to demonstrate inhibition of epinephrine-induced lipolysis (15) by NGF. Glycerol release (2 hr, 37°) was stimulated 5-fold by *l*-norepinephrine (20 ng/ml), and this response was 90% inhibited by insulin (1 ng/ml). The β-subunit of NGF caused no inhibition in concentrations varying from 10 ng/ml to 20 μg/ml. The 7S form of NGF also had no effects at concentrations less than 1 μg/ml; some inhibition, however, was demonstrated with 2 μg/ml (20%), 4 μg/ml (28%), and 10 μg/ml (32%). NGF preparations had no significant lipolytic activity.

DISCUSSION

Binding of [^{125}I]NGF to membrane fractions of superior cervical ganglia of rabbits appears to represent an interaction with the physiological receptor for NGF. Of the various tissues examined, only preparations of superior cervical ganglia display specific NGF binding. The binding is a saturable process over the concentration range of NGF that exists in the circulation and that is biologically effective in assays *in vivo*. Furthermore, the dissociation constant (about 0.2 nM) calculated from the kinetics of binding corresponds closely to that estimated from bioassays.

NGF binding is not modified by high concentrations of several other peptides, including proinsulin, whose amino-acid sequence bears similarities to NGF (3, 4), or epidermal growth factor, another growth-promoting hormone which, like NGF, is produced by salivary glands of mice. The close correlation of biological activity with receptor binding displayed by NGF samples treated with *N*-bromosuccinimide provides further evidence that the [^{125}I]NGF binding observed here represents true receptor binding.

The binding assay used in the present study is simple, sensitive, and specific. It provides an efficient means for examining biological specimens for NGF-receptor interactions and might be useful in studies of pathological states, such as hyperthyroidism, hypertension, and malignant disease. We recently observed that human lymphocytes stimulated with concanavalin-A develop NGF receptors (manuscript in preparation) coincident with the emergence of insulin receptors (16).

Like receptors for many hormones, the NGF receptors are localized to membrane fragments and may be present on the cell surface. The bimolecular nature of the association constant suggests that NGF binds to a single species of a receptor. The dissociation constant calculated from the ratio of k_{-1}/k_1 (0.23 nM) is similar to the value of 0.2 nM calculated on the basis of the concentrations of free NGF and receptor and of their complex at equilibrium under nonsaturating conditions. This value is quite similar to plasma concentrations of NGF in mouse serum, which are 0.4 nM (17).

The recent demonstration that somatomedin ("sulfation factor", "thymidine factor"), a peptide hormone that has potent insulin-like activity on isolated fat cells, can effectively compete with [^{125}I]insulin for binding to receptors in various tissues (18) has raised the possibility that other basically anabolic and growth-promoting hormones may possess some common receptor properties. This possibility seemed especially important for NGF since this hormone has metabolic effects on sympathetic ganglia that are similar to those of insulin on its target tissues, and since the primary structure of NGF has certain analogies to that of proinsulin, a hormone that activates insulin responses by binding to insulin receptors (19). Our demonstration in these studies that proinsulin and insulin do not affect the binding of [^{125}I]NGF, that NGF does not alter the binding of [^{125}I]insulin, and that NGF does not elicit insulin-like effects in adipocytes indicates that the receptors for insulin and for NGF are highly specific and possess little or no cross reactivity, with respect to specificity. The receptors for NGF and for epidermal growth factor also do not demonstrate cross-specificity. Furthermore, no cross reactivity exists between the receptors for insulin and for epidermal growth factor (manuscript in preparation).

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