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Modulation of the Neuronal Binding of the β Subunit of Nerve Growth Factor (NGF) by the α-NGF Subunit

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The effect of the α subunit of the 7S-NGF on the binding of β-NGF to its two classes of sites on target cells has been studied. The presence of μM concentrations of α-NGF causes the displacement of ^{125}I-β-NGF from one class of sites on dissociated dorsal root ganglia neurons from stage E9 chicken embryos. At 0.1 nM ^{125}I-β-NGF, increasing α-NGF concentrations produce a monotonic displacement curve with half-maximal displacement occurring at 10 μM α-NGF. The affinity and number of sites of the ^{125}I-β-NGF displaced by α-NGF are similar to those of β-NGF that binds to the higher affinity (site I) receptors. The binding to the lower affinity class of sites (site II) is not affected by concentrations of α-NGF up to 30 μM. This modulation of ^{125}I-β-NGF binding does not occur with equivalent concentrations of serum albumin. No detectable neuronal binding of ^{125}I-α-NGF was found, suggesting that the mechanism does not involve direct competition for receptor sites. The dissociation constant for the α-β complex is in the μM range, and formation of this complex in solution can thus compete with the process of ^{125}I-β-NGF binding to neurons. A model accounting for these observations includes binding of the α-β complex to the lower affinity but not to the higher affinity sites. We conclude that there are differences in the specificity of the two classes of receptors.

Key words: nerve growth factor, alpha subunit, neuronal binding, NGF receptors, dorsal root ganglia

Nerve growth factor is an important protein involved in the development, maintenance, and regeneration of sympathetic and sensory neurons. The 7S-NGF, the oligomeric form found in all the richest mammalian sources including the male mouse submaxillary gland, the guinea pig prostate, and the human placenta, is composed of three distinct classes of subunits; α-, β- and γ-NGF. The characteristic biological activities of mouse NGF reside with the beta subunit, whereas the gamma subunit possesses an esterase activity which may function to process a precursor form of β-NGF to the biologically active species, but no definable activity, enzymatic or biological, has been discovered for the alpha subunit.

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The best current model for the subunit stoichiometry and arrangement of the mouse 7S NGF is an $\alpha_2\beta\gamma_2$ structure. Various intermediate complexes are found upon the dissociation of the 7S oligomer depending on the specific conditions [1]. The results from a number of groups [1–4] suggest that the alpha and gamma subunits do not interact to form a complex, whereas either alpha or gamma interact with the beta subunit to form a specific complex.

At equilibrium, Scatchard analysis of the binding of isolated β-NGF to whole sensory neurons results in curvilinear plots [5–7]. Evidence presented by Sutter et al [7] suggests that this heterogeneity in binding is due to two distinct classes of binding sites, referred to as site I and site II; however, evidence has been presented that the curvilinearity cannot be attributed to independent sites but rather may involve negatively cooperative interactions [6, 7]. The dissociation constant of site I, the higher affinity site, corresponds to the concentration required for half-maximal stimulation of neurite outgrowth whereas the lower affinity site has about a 100-fold weaker affinity for β-NGF and has an uncertain biological function.

Stach and Shooter [8] examined a chemically cross-linked 7S-NGF species for its ability to bind to neuronal receptors, but found no binding. The elimination of specific binding cannot, however, be taken as evidence that the native 7S oligomer does not bind since no reversible cross-linking procedure was used to demonstrate that the β-NGF remains biologically intact. Harris-Warrick et al [9] utilized another approach to this same problem. They attempted to ensure that the labeled β-NGF used in their binding assays was completely complexed by the addition of excess alpha and gamma subunits as well as zinc ion, which further stabilizes the 7S complex. Their examination of the effect of the α-NGF subunit on β-NGF cellular binding, however, was not extended to the concentration range in which previous investigators have reported the α-β complex dissociation constant. Therefore we have further examined the effects of added α-NGF on the binding properties of β-NGF.

MATERIALS AND METHODS

Preparation of Nerve Growth Factor Subunits

The NGF subunits were prepared from adult male mouse submaxillary glands by the initial purification of the 7S-NGF protein by the method of Stach et al [10], followed by separation of the acid dissociated subunits by chromatography on CM-cellulose following the method of Smith et al [11]. The isolated subunits were routinely examined by isoelectric focusing in polyacrylamide gels for purity. The α-NGF subunit was additionally rechromatographed on another CM-cellulose column to ensure its homogeneity.

Iodination

β-NGF was radiolabeled with ^{125}I by the method of Sutter et al [7] utilizing lactoperoxidase and purified by Amicon CF-50A Centriflo filtration. This same procedure was followed for the radioiodination of α-NGF except for the substitution of sodium phosphate–potassium phosphate buffer ($I = 0.05$) at pH 6.8 for the pH 4.0 sodium acetate buffer used for β-NGF.

Ganglia Dissociation

Dorsal root ganglia were dissected out of stage E9 chicken embryos and collected in cold calcium- and magnesium-free phosphate-buffered Gey's balanced salt solution, pH 7.4 (CMF-PBG). Following washing of the whole ganglia by centrifugation through the same buffer, digestion was initiated by the addition of trypsin (Difco 1:250) to 0.25%. After incubation at 37°C for 20 min the ganglia were centrifuged and washed twice with phosphate buffered Gey's balanced salt solution containing 2 mg/ml bovine serum albumin (BSA-PBG). Dissociation was carried out by gentle tritration with a small gauge needle. These cells were washed in BSA-PBG twice, and then the larger clumps were removed by passage through a nylon mesh (30 μm). Trypan blue excluding cells were counted in a hemocytometer.

Binding Assay

The specific binding to the dissociated sensory neurons was measured as described by Sutter et al [7]. This method utilizes centrifugation at 10,000g of a 100-μl sample through 175 μl of BSA-PBG containing 0.15 M sucrose to separate bound from free. The 400-μl microfuge tubes were then frozen in liquid nitrogen, the bottom 2 mm containing the pellet sliced off, and both portions were counted in a Packard gamma counter.

Measurement of α-β Complex Formation

The extent of complex formation was assayed by the separation of complexed from free ^{125}I-β-NGF on microcolumns of CM-cellulose, in a similar fashion to that described by Harris-Warrick et al [9] except that centrifugation rather than compressed air was used for elution.

Preparation of Bisdes-Arg^{118} β-NGF

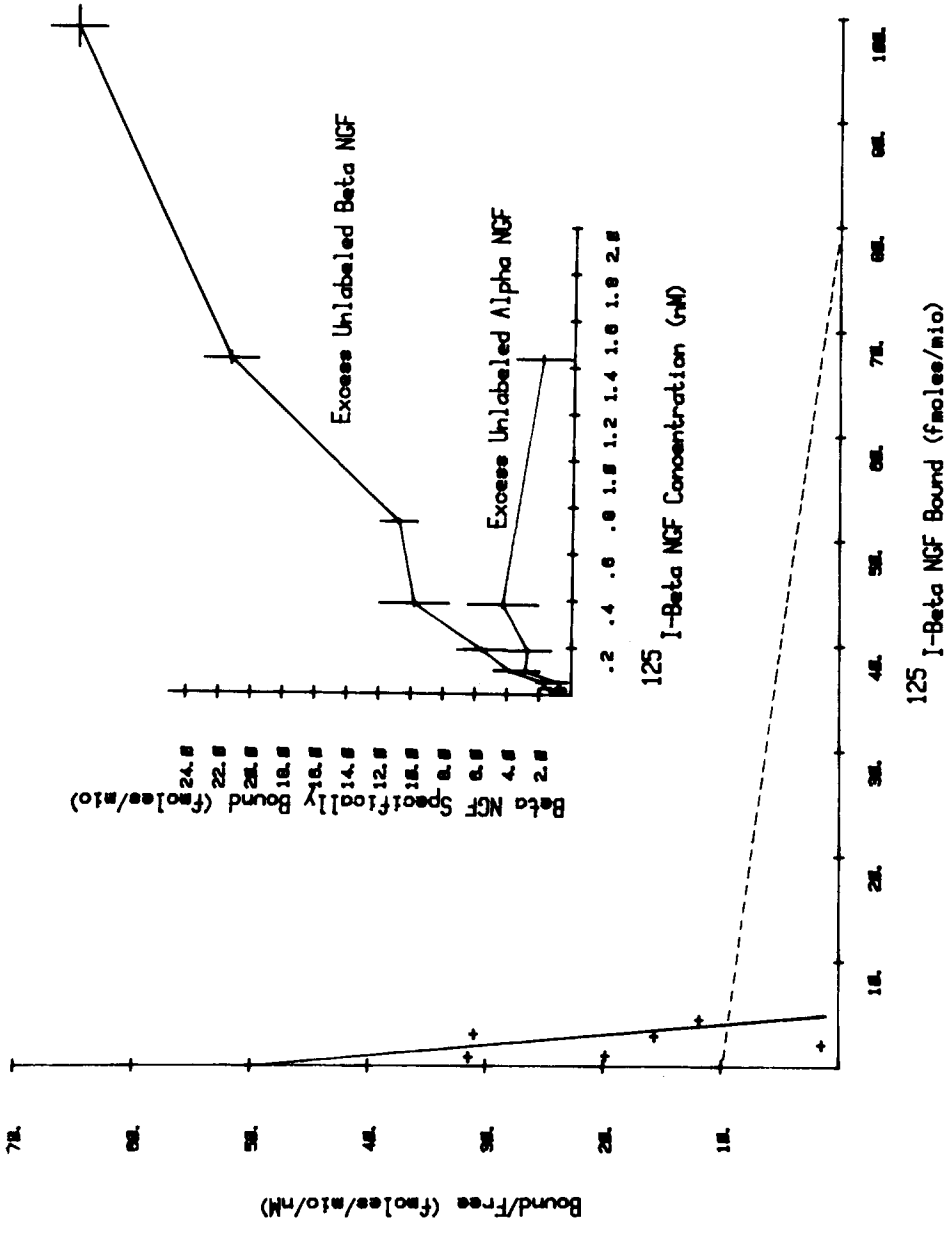
The procedure of Moore et al [12] was used to remove the carboxy-terminal arginine residues by the limited proteolytic digestion with carboxypeptidase B.

Isoelectric Focusing

Isoelectric focusing in polyacrylamide gels as described by Greene et al [13] was used to establish the extent of bisdes-Arg^{118}β-NGF formation.

RESULTS

The addition of a high concentration of unlabeled α-NGF to a labeled β-NGF binding assay produces different effects on the interaction of β-NGF with cells depending on the concentration of β-NGF present. Under low labeled β-NGF concentration conditions (allowing mainly the high affinity receptors to effectively bind β-NGF), the presence of the α-NGF subunit is able to mimic the effect produced by an excess of unlabeled β-NGF, whereas at higher concentrations of labeled β-NGF (which will be increasingly bound to low affinity sites) this ability is lost. Experimentally, the result is that shown by the graphs of Figure 1.


Bound/Free (fmolee/mio/nM)
125 I-Beta NGF Bound (fmolee/mio)
125 I-Beta NGF Concentration (nM)
Excess Unlabeled Beta NGF
Excess Unlabeled Alpha NGF
Beta NGF Specifically Bound (fmolee/mio)

The labeled β-NGF was incubated with cells both alone and in the presence of either 6 μM unlabeled β-NGF or 30 μM unlabeled α-NGF, and the resulting points (inset) are the differences between the total (β-NGF alone) and the experimental incubations. Although the curves are not statistically different at the low labeled β-NGF concentrations, the curves progressively deviate at higher concentrations. This displacement data is also plotted by the method of Scatchard in Figure 1. The solid line is the high affinity component (K_D = 0.1 nM with 3,000 sites per cell), and the dashed line is the lower affinity component (K_D = 8 nM with 50,000 sites per cell), both from the biphasic curve (not shown) describing the β-NGF binding, and the data points are the "specific" β-NGF binding as defined by the displacement caused by the excess α-NGF . The graph illustrates that the labeled β-NGF that could be displaced by 30 μM α-NGF has an apparent affinity and number of binding sites similar to the high affinity binding component, and that 30 μM α-NGF is apparently unable to cause the displacement of labeled α-NGF from the lower affinity sites.

This interaction was also examined at a fixed β-NGF concentration of 0.1 nM, a concentration that would be expected to have most of the bound β-NGF filling high affinity sites. The displacement of labeled β-NGF was followed through a series of unlabeled α-NGF concentrations, and the resulting displacement curve (Figure 2) shows an apparently monotonic curve with a midpoint at 10 μM α-NGF subunit added. Addition of unlabeled α-NGF produced almost a complete displacement of bound labeled β-NGF at about 30 μM.

The direct competition by α-NGF for the high affinity β-NGF receptors, but not for the low affinity sites, would adequately explain these data. Experiments were performed (Figure 3) with the dorsal root ganglia preparations and have indicated that ^{125}I-α-NGF in the concentration range of 4 to 40 nM does not interact in a specific and saturable fashion. Assuming that if α-NGF bound specifically and in a saturable fashion, and if about the same total number of cellular sites might be expected as is found for β-NGF, calculation of the proportion of binding which would result from the nonspecific component indicates that the current methods would not be sensitive enough to detect such a specific component occurring at 10–30 μM free radiolabeled α-NGF concentrations.

The carboxypeptidase B digested β-NGF, bisdes-Arg^{118}β-NGF, has been reported to be unable to reform the 7S-NGF complex with the addition of α-NGF and

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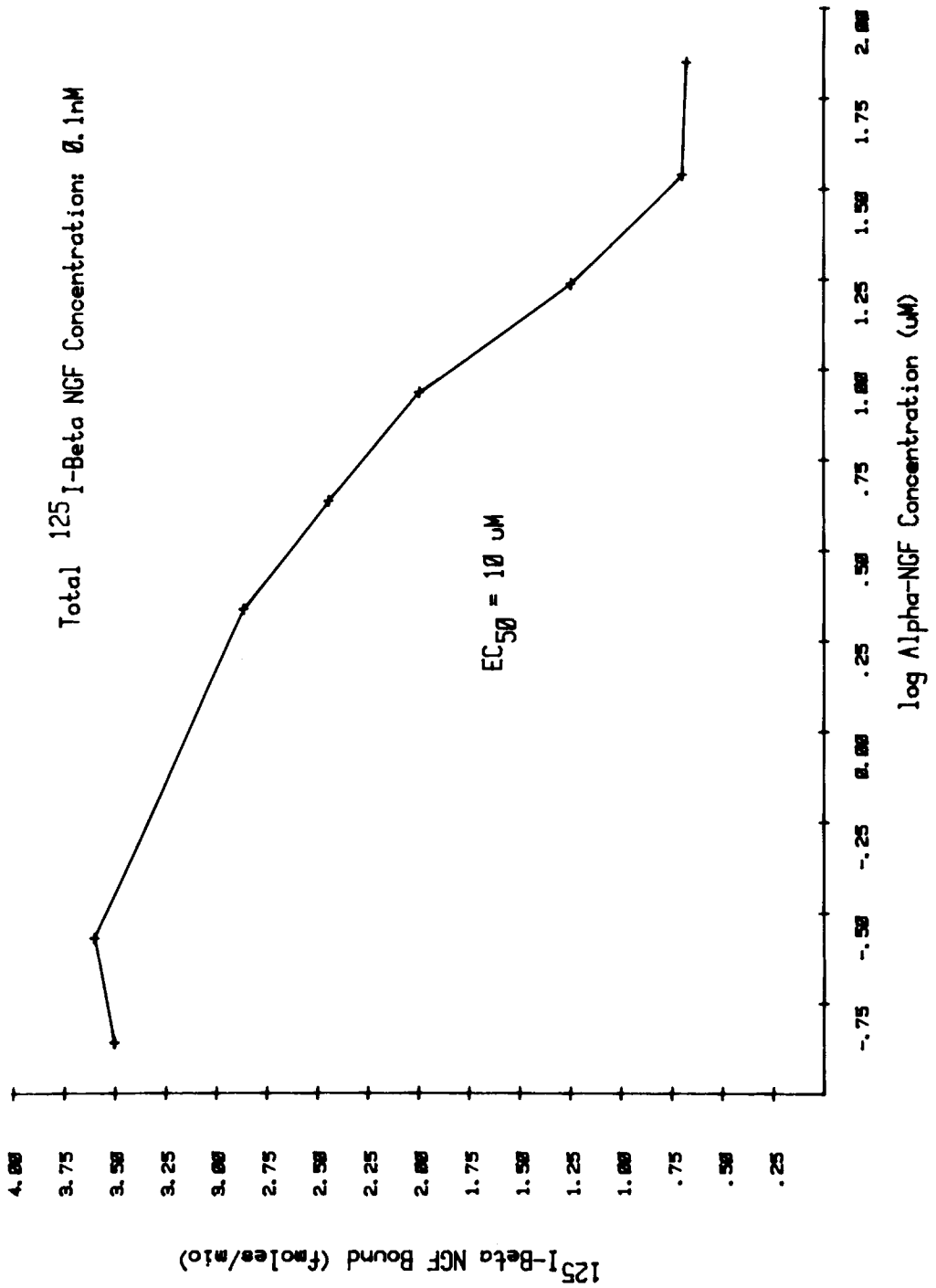
Fig. 1. ^{125}I-β-NGF binding to dissociated sensory neurons and displacement by unlabeled β-NGF or by unlabeled α-NGF as plotted according to Scatchard or as bound versus free (inset). Three parallel incubations were carried out at 37°C for 45 min with a series of labeled β-NGF concentrations (10 pM to 2 nM) and contained dorsal root ganglia neurons at a final concentration of 0.80 million cells/ml. Excess unlabeled β-NGF (6 μM) was added to the cell incubation (in the first set of tubes), the nonspecifically bound labeled α-NGF remaining was subtracted from the total binding (assayed in the second set of tubes without further additions), and the resultant specifically bound (or displaced) β-NGF was plotted (mean ± SD of triplicates) as indicated by "excess unlabeled β-NGF." Then 30 μM α-NGF was added to the third set of tubes, the labeled β-NGF that remained bound was subtracted from the total binding, and the difference (corresponding to a component specifically displaced by α-NGF) was plotted (mean ± SD of triplicates) as indicated by "excess unlabeled α-NGF." The data points (+) in the Scatchard plot were obtained from the later experiment as the ^{125}I-β-NGF displaced by 30 μM α-NGF; the solid line and the dashed line represent the high affinity (K_D = 0.1 nM, 3,000 sites/cell) and the low affinity (K_D = 8 nM, 50,000 sites/cell) components from the biphasic curve of a more extensive study of specific (displaced by 6 μM unlabeled β-NGF) β-NGF binding.

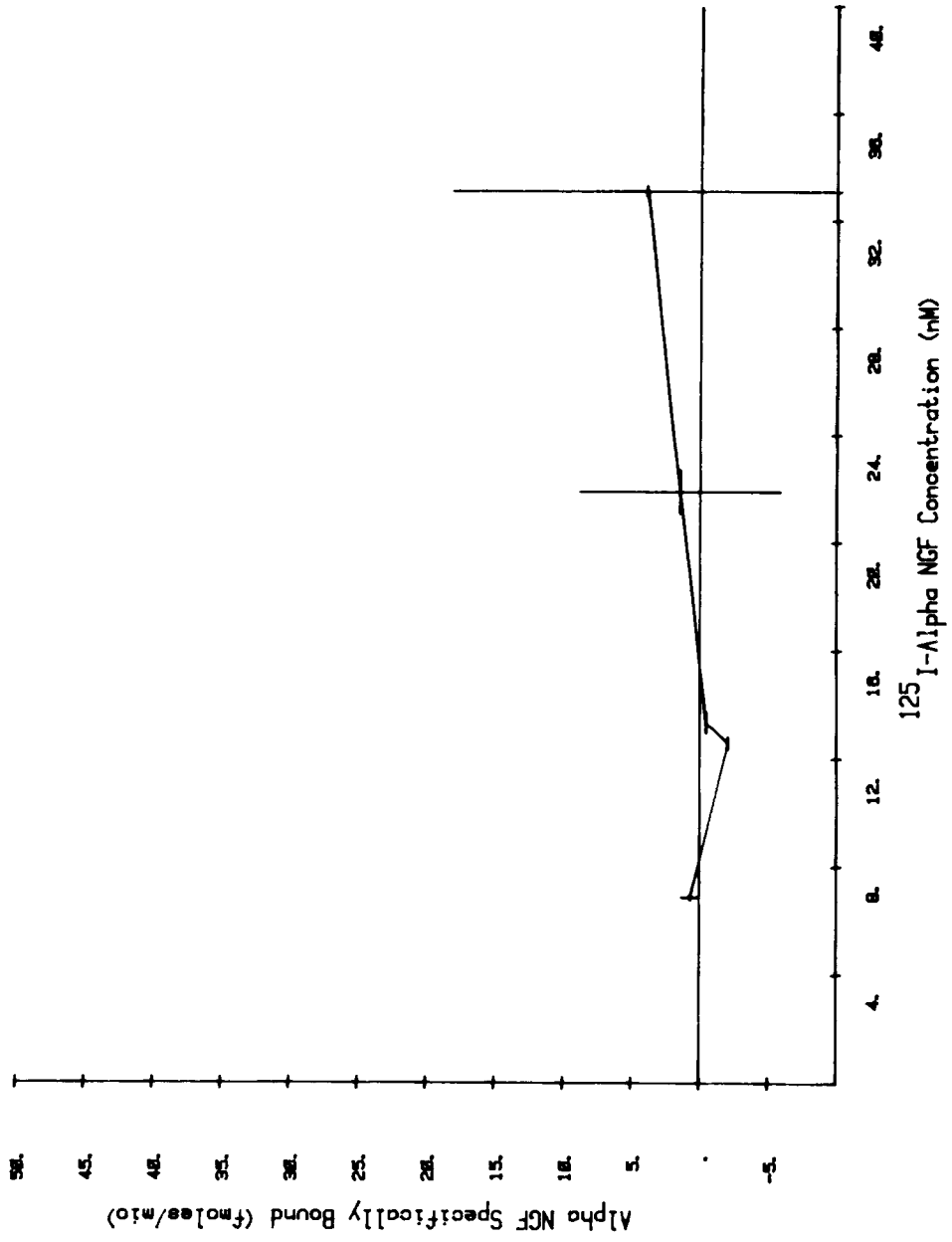

Total 125I-Beta NGF Concentration: 0.1nM
EC50 = 10 uM
125I-Beta NGF Bound (fmoles/mio)
log Alpha-NGF Concentration (uM)
4.00
3.75
3.50
3.25
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
.75
.50
.25
-.75
-.50
-.25
0.00
.25
.50
.75
1.00
1.25
1.50
1.75
2.00

γ-NGF subunits [12]. This proteolyzed form of β-NGF was produced using the protocol of Moore et al [12], and examined with the dissociated dorsal root neuron binding assay for the ability to be displaced from the high affinity binding sites by the α-NGF subunit. The results indicated no significant difference between parallel assays using undigested β-NGF. At 0.1 nM ^{125}I-β-NGF, 30 μM α-NGF displaced 4.5 ± 1.5 fmoles of bound label per million cells, and, at the same concentration of ^{125}I-bisdes-Arg^{118}β-NGF, 3.0 ± 0.7 fmoles was also displaced by that same concentration of α-NGF. Isoelectric focusing in polyacrylamide gels was carried out and clearly demonstrated that the conversion to bisdes-Arg^{118}β-NGF was complete. To verify that α-β complex formation was prevented by this digestion, under our binding conditions, assays were performed utilizing the ability of CM-cellulose to bind both free β-NGF and bisdes-Arg^{118}β-NGF, while not retaining the less basic complexes with the α-NGF subunit [3]. Samples of radioiodinated bisdes-Arg^{118}β-NGF and undigested β-NGF (both at 10 nM) were incubated in the presence and absence of 30 μM α-NGF in a phosphate buffer (pH 6.8) containing 1 mg/ml bovine serum albumin. Aliquots of each were simultaneously applied to small columns of CM-cellulose (300 μl of resin in a 1-cc tuberculin syringe) equilibrated in the same buffer. These were centrifuged and washed twice with 200 μl of buffer. Without the addition of α-NGF, 91% of the ^{125}I-β-NGF and 88% of the bisdes-Arg^{118}β-NGF were retained by the resin. In the presence of 30 μM α-NGF, only 29% of the β-NGF was retained, as expected; however, in the presence of the α-NGF subunit, only 28% of the bisdes-Arg^{118}β-NGF was retained, thus demonstrating that under these conditions of high α-NGF concentration both β-NGF species were capable of forming complexes with α-NGF. Thus, the lack of binding of bisdes-Arg^{118}β-NGF to high affinity receptors in the presence of α-NGF is probably due to complex formation.

DISCUSSION

We have demonstrated that the addition of the α-NGF subunit to radioiodinated β-NGF binding assays modulates the interaction with dissociated dorsal root neurons. The quantitative aspects of these effects strongly suggest that binding of ^{125}I-β-NGF to its high affinity receptor is completely blocked whereas binding to the low affinity site is apparently unaffected. We have investigated two mechanisms by which the data can be simply, yet adequately, explained. First, that addition of α-NGF promotes formation of an α-β complex in solution which has different binding properties to the two neuronal receptor classes. Second, that the inclusion of α-NGF in the binding assay is competing with the labeled β-NGF for the high affinity sites by a direct interaction with the neurons. Two lines of evidence suggest that the first mechanism is most likely. The labeled α-NGF direct binding experiments we presented have not revealed any specific binding, although for the technical reasons described, radiola-

Fig. 2. Competition curve of α-NGF for ^{125}I-β-NGF binding to dissociated sensory neurons. The incubation mixture consisted of dorsal root ganglia neurons at a final concentration of 0.91 million cells/ml, labeled β-NGF at 0.10 nM, and a series of α-NGF concentrations ranging from 70 nM to 70 μM. Nonspecific binding, as estimated by utilizing an excess of unlabeled β-NGF, was 0.75 fmoles/million cells (mio). The concentration of labeled β-NGF used here means that approximately 72% of the total bound β-NGF occupied the high affinity sites, using constants derived from previous experiments.


Alpha NGF Specifically Bound (fmoles/mio)
50.
45.
40.
35.
30.
25.
20.
15.
10.
5.
0.
-5.
4.
8.
12.
16.
20.
24.
28.
32.
36.
40.
125I-Alpha NGF Concentration (nM)

beled α-NGF concentrations larger than those examined will produce a nonspecific binding component that would mask any specific component the size of the high affinity β-NGF binding class. More importantly, we have found that the concentration of α-NGF which produces a half-maximal effect (about 10 μM in our studies) corresponds very closely with the dissociation constant for the α-β complex in solution, reported to be in the low μM range by Palmer and Neet [2] and as 5 μM by Almon and Varon [3].

Our conclusion that the high and low affinity receptors have a different specificity for the α-β complex is not affected by the particular mechanism for receptor sites. Physically heterogeneous sites [7] could clearly discriminate the β-NGF subunit from the α-β complex sterically, whereas negative cooperativity [5, 6] or converison [14] between sites could be altered by the binding of β-NGF into the α-β complex.

A model which is constant with these data is illustrated in Figure 4. R1 and R2 are the high affinity and lower affinity β-NGF binding sites, respectively. The β-NGF subunit is shown to be able to bind to each site, and is designated by a line connecting them. The line connecting the β-NGF subunit with the α-β complex is meant to

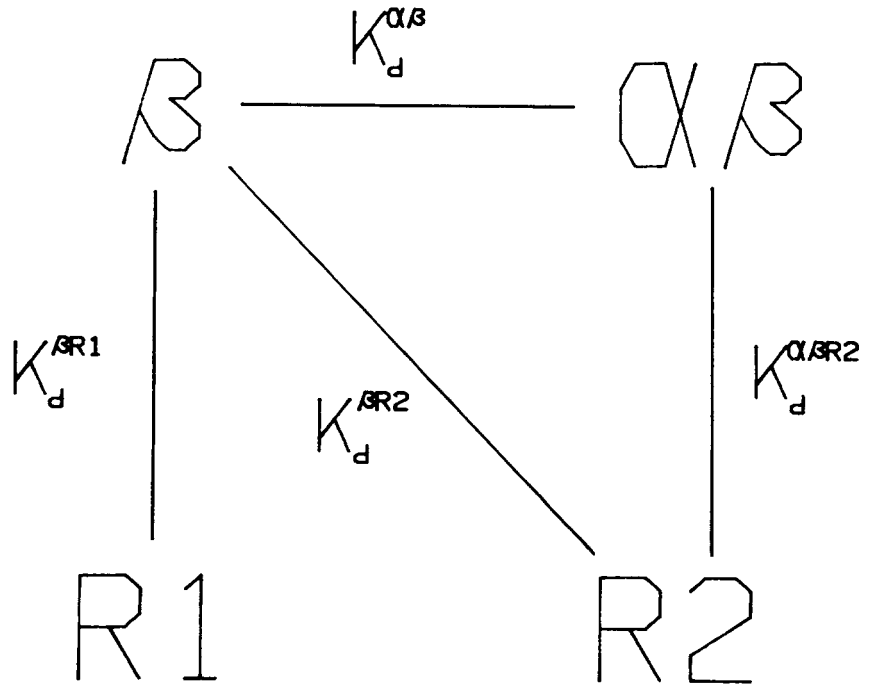


Fig. 4. Model for the binding of β-NGF and the α-β complex to the high affinity (R1) and lower affinity (R2) receptors on dorsal root ganglia neurons.

Fig. 3. Lack of binding of ^{125}I-α-NGF to dissociated sensory neurons. The incubations contained dorsal root ganglia neurons at a final concentration of 1.3 million cells/ml. Incubation with the radiolabeled α-NGF subunit was allowed to proceed for 45 min at 37°C, plus or minus an excess (greater than 1,000-fold) of unlabeled α-NGF subunit to estimate the nonspecific binding. Error bars indicate standard deviation of triplicate measurements.

indicate their equilibrium in solution. We suggest that whereas the α-β complex is able to bind to the lower affinity sites, indicated by the line to R2, it is not able to bind to the high affinity receptors, R1.

Greene and Shooter [15] have suggested that neurite outgrowth corresponds to the binding of β-NGF to its high affinity receptor since the half-maximal concentration effective in the bioassay is the same as the dissociation constant determined from Scatchard analysis for the binding to the high affinity site. This conclusion is reasonable since the two sites differ in affinity by about 100-fold, and since mediation of the biological response by the lower affinity receptor would entail an unreasonably low occupancy. Cellular responses other than neurite outgrowth, however, occur over concentration ranges that do not eliminate the site II receptors from consideration [15]. Up until now, examination of the biological consequences of β-NGF binding to site II, in the absence of site I binding, has been impossible. Our results suggest, however, that via complex formation with α-NGF, we will be able to probe the function of the class of site II receptors.

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REFERENCES

1. Palmer TE, Neet KE: J Biol Chem 255:5170, 1980.
2. Palmer TE, Neet KE: Arch Biochem Biophys 205:412, 1980.
3. Almon RR, Varon S: J Neurochem 30:1559, 1978.
4. Silverman RE: PhD thesis, Washington University, St. Louis, 1977.
5. Frazier WA, Boyd LF, Bradshaw RA: J Biol Chem 249:5513, 1974.
6. Frazier WA, Boyd LF, Pulliam MW, Szutowicz A, Bradshaw RA: J Biol Chem 249:5918, 1974.
7. Sutter A, Riopelle RJ, Harris-Warrick RM, Shooter EM: J Biol Chem 254:5972, 1979.
8. Stach RW, Shooter EM: J Neurochem 34:1499, 1980.
9. Harris-Warrick RM, Bothwell MA, Shooter EM: J Biol Chem 255:11284, 1980.
10. Stach RW, Wagner BJ, Stach BM: Anal Biochem 83:26, 1977.
11. Smith AP, Greene LA, Fisk HR, Varon S, Shooter EM: Biochemistry 8:4918, 1969.
12. Moore JB, Mobley WC, Shooter EM: Biochemistry 13:833, 1974.
13. Green EA, Varon S, Piltch A, Shooter EM: Neurobiology 1:37, 1971.
14. Landreth RS, Shooter EM: Proc Natl Acad Sci USA 77:4751, 1980.
15. Greene LA, Shooter EM: Ann Rev Neurosci 3:353, 1980.