Inhibition of β Nerve Growth Factor Binding to PC12 Cells by α Nerve Growth Factor and γ Nerve Growth Factor†

Nathaniel R. Woodruff and Kenneth E. Neet*

Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

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ABSTRACT: Pheochromocytoma (PC12) cells have been found to differ from dorsal root ganglionic cells with respect to the modulation of the β nerve growth factor (βNGF) binding properties elicited by αNGF and γNGF. In contrast to our previous results with intact dorsal root ganglionic cells in which only high-affinity binding was blocked, αNGF and γNGF were found to block competitively all steady-state binding of iodinated βNGF to PC12 cells at both 37 and 0.5 °C. The EC_{50} that was found for the αNGF displacement was 9–10 μM, and the γNGF effect had an EC_{50} of 200 nM, in the predicted range based upon the apparent K_d for dissociation of the αβ or the βγ complex in solution. The concurrence of the binding EC_{50} and the K_d for each complex indicates that the formation of αβ or βγ complexes in solution competes with the process of PC12 receptor binding with ^{125}I-βNGF. Experiments were carried out examining the dissociation kinetics following the addition of excess unlabeled βNGF or αNGF at both 37 and 0.5 °C. Three dissociation components were observed with αNGF, in contrast to the two normally found with βNGF. Lowering the chase temperature to 0.5 °C changed the relative contributions made by each component without dramatically changing any of the rate constants. The "slow" receptor was further examined by the dependence on ^{125}I-βNGF concentration of the slowest component with a chase of either excess αNGF or excess γNGF at 0.5 °C. These cold chase stable sites include a positively cooperative class of sites (3000–4000 sites/cell) that exhibit first and second stoichiometric equilibrium constants of about 50 pM and 5–10 pM, respectively. These sites demonstrate close similarity to the unlabeled βNGF dissociation–chase results both quantitatively and qualitatively. Thus, the cold chase protocol does not appear to be influenced by binding of excess βNGF to additional receptor sites but is a property of the binding sites themselves. The findings contrast with those from dorsal root ganglionic cells and thus imply differences in the NGF receptor systems between these primary cells and the clonal PC12 cell line.

The 7S oligomeric form of NGF[1] dissociates below micromolar concentrations (Almon & Varon, 1978; Bothwell & Shooter, 1978; Palmer & Neet, 1980a,b; Silverman & Bradshaw, 1982; Rao & Neet, 1984; Nichols & Shooter, 1983) such that the βNGF dimer is sufficient for biological activity (Greene & Shooter, 1980). Under certain conditions, however, the α and γ subunits have been shown to modulate the interaction of βNGF with neuronal cells. Stach and Shooter (1980) showed that a cross-linked 7S NGF was biologically inactive with the sensory neuron bioassay. Harris-Warrick et al. (1980) demonstrated convincingly that the 7S oligomer in the presence of excess Zn^{2+}, αNGF, and γNGF did not bind to either high-affinity or low-affinity receptors on sensory neurons. These workers also showed that the γ subunit, by itself, was a competitive inhibitor of ^{125}I-βNGF binding to the high-affinity receptor but could demonstrate no effect of 0.4 μM αNGF on βNGF binding to the neurons. Woodruff and Neet (1982) extended these studies with micromolar concentrations of αNGF where αβ complex formation would predominate and concluded that the αβ complex does not bind to the high-affinity receptor (K_d = 0.1 nM) of dorsal root ganglia neurons but still retains its ability to bind to low-affinity receptors (K_d = 8 nM).

Prior to the work presented here, no reports have appeared concerning the effects of the other 7S NGF subunits on the binding properties of βNGF to PC12 cells. Distinct differences in βNGF binding properties exist between the ganglionic whole cell system and the PC12 cells, which suggests that the αNGF and γNGF subunits may also prove to be useful in the elucidation of important relationships between these two cell systems. This approach is especially significant due to the varying reports in the literature on the steady-state binding properties of the PC12 clonal cell line (Schechter & Bothwell, 1981; Landreth & Shooter, 1980; Yankner & Shooter, 1979; Bernd & Greene, 1984). Two kinetic components of receptors have been reported in PC12 cells (Schechter & Bothwell, 1981; Landreth & Shooter, 1980), although they have been interpreted as either preexisting receptors (Schechter & Bothwell, 1981) or an NGF-induced conversion between rapidly and slowly dissociating receptors (Landreth & Shooter, 1980). Conversion between fast and slow receptors has also been accomplished by fusion of PC12 plasma membrane fragments into 3T3 cells (Block & Bothwell, 1983), by lectins (Grob & Bothwell, 1983; Vale & Shooter, 1982), and by monoclonal antibodies (Vale & Shooter, 1983). Whether the slow receptor represents internalized or sequestered receptor (Yankner & Shooter, 1979; Bernd & Greene, 1984; Chandler & Herschman, 1983; Calissano & Shelanski, 1980; Layer & Shooter,

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* Author to whom correspondence should be addressed.

[1] Abbreviations: PBS, Dulbecco's phosphate-buffered saline; CMF-PBS, calcium- and magnesium-free Dulbecco's phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; βNGF, β subunit of nerve growth factor; αNGF, α subunit of nerve growth factor; γNGF, γ subunit of nerve growth factor; PC12, pheochromocytoma 12 cell line; BSA, bovine serum albumin; RMS, root mean square; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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1983) or a different form of a cell surface receptor (Landreth & Shooter, 1979) has also been debated. Results agree, however, that the cold chase stable or slow receptor bound βNGF is at least partially, depending on conditions, a plasma membrane receptor bound form of ligand. In the preceding paper (Woodruff & Neet, 1986) we have reported that these cold chase stable sites are composed of a single small class of positively cooperative sites under conditions with little internalization. The cooperative interactions occur within the concentration range responsible for biological activity, which may provide a mechanism by which the cell ensures a higher level of occupancy at the low physiological concentrations.

Since the kinetic and steady-state properties of the two different classes of PC12 cell βNGF receptors are complex, we have utilized the properties of the other two NGF subunits, α and γ, to elicit additional distinguishing properties and to initiate a refinement of the structural requirements for receptor binding of βNGF. The results suggest that αβ or βγ complexes do not bind to or differentiate between the βNGF receptors of PC12 cells.

Materials and Methods

Tris-HCl buffer contained 50 mM Trizma, pH 7.4 at 25 °C. Sodium/potassium phosphate buffer contained 15.7 mM KH_2PO_4 and 11.4 mM Na_2PO_4, pH 6.8, with an ionic strength of 0.05. PBS (Dulbecco's phosphate-buffered saline) and CMF-PBS (calcium- and magnesium-free Dulbecco's phosphate-buffered saline) were obtained from GIBCO as a powder and mixed according to the manufacturer's instructions. PBS/BSA solution was pH 7.4 PBS made to contain 1 mg/mL BSA (Pentex crystalline) and sterilized. DMEM (Dulbecco's modified Eagle's medium) was obtained from GIBCO; complete DMEM was prepared by the addition of 5% v/v of fetal bovine serum (GIBCO) to DMEM. Binding PBS was Dulbecco's PBS containing 1 mg/mL BSA and 1 mg/mL glucose, pH 7.4. Binding assay sucrose cushion buffer was PBS made 0.15 M in sucrose (Schwarz/Mann, ultrapure, density gradient grade).

Isolation of 7S NGF was carried out by the method of Stach et al. (1977) with modifications as described previously (Woodruff & Neet, 1986). In order to ensure very high purity of the αNGF and γNGF subunits, they were separately rechromatographed on new CM-cellulose (Whatman CM52). For αNGF, this procedure was identical with the original subunit preparation, and the impurities, particularly contaminating γNGF, were retained on the column while the αNGF peak was collected and reconcentrated by vacuum dialysis. The γNGF rechromatography was carried out in a similar fashion except that the wash with the αNGF elution buffer was discarded.

PC12 cells (Greene & Tischler, 1976) were grown in complete DMEM as described (Woodruff & Neet, 1986; Greene & Tischler, 1976). Biological assays of NGF activity were performed with the PC clonal cell line (Landreth & Shooter, 1980; Greene, 1977; Black & Greene, 1982). Response of cells was always 70–80% or better with a half-maximal activity at about 25 pM βNGF. The protocol for the βNGF iodination reaction was the same as that described by Sutter et al. (1979) with the modification and characterization of ^{125}I-βNGF as described previously (Woodruff & Neet, 1986). γ counting was done in a scintillation counter (Packard Model 5360 Modumatic VI) with semiautomatic data acquisition as described (Woodruff & Neet, 1986). The binding assay followed the one-step sucrose sedimentation protocol described by Sutter et al. (1979) with modifications and data treatment as described previously (Woodruff & Neet, 1986). Subsequent analysis of binding data utilized the LIGAND program (Munson, 1983) provided by Dr. Rodbard and modified to run on a DEC System 20. The multiterm Scatchard equation with or without bivalent cooperativity and with or without a nonspecific binding term was utilized as described previously (Woodruff & Neet, 1986). The dissociation kinetics of ^{125}I-βNGF were assayed following a 30-min incubation at 37 °C with PC12 cells. Dissociation was initiated by addition of unlabeled βNGF, αNGF, or γNGF either immediately at 37 °C or after cooling for 5 min at 0.5 °C. Data were obtained at various times, analyzed, and fit (Mannervick, 1982) to a multiterm exponential as described previously (Woodruff & Neet, 1986).

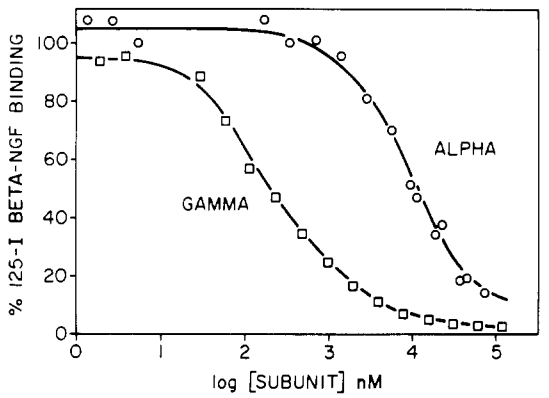

FIGURE 1: αNGF and γNGF competition curves. Apparent competition for ^{125}I-βNGF binding to PC12 cells by increasing concentrations of αNGF (○) or γNGF (□). The labelled βNGF concentration was fixed at 1.05 ± 0.06 nM, and the PC12 cells were at a final assay concentration of 1.1 million cells/mL. The incubation was initiated by the addition of cells to the subunit mixture and was continued for 40 min at 37 °C. The specific binding of ^{125}I-βNGF in the absence of other subunits was 118.5 ± 1.8 fmol/million cells.

Results

Inhibition Experiments with αNGF and γNGF. The effect of the presence of αNGF on the binding of ^{125}I-βNGF to PC12 cells was initially examined with competition experiments at 37 °C with a fixed ^{125}I-βNGF concentration. Increasing concentrations of αNGF decreased the total binding of 1 nM ^{125}I-βNGF (Figure 1). The curve thus generated was monotonic with an EC_{50} of 9–10 μM αNGF and nearly complete inhibition of ^{125}I-βNGF specific binding.

The results of steady-state experiments performed to examine the influence of γNGF were qualitatively very similar to those of the αNGF experiments; however, some of the quantitative aspects of these interactions differed. At fixed ^{125}I-βNGF concentration increasing concentrations of unlabeled γNGF coincubated with PC12 cells at 37 °C produced decreasing binding (Figure 1). The resulting curve was monotonic, as was observed for αNGF, but the effect occurred with an EC_{50} of 200 nM, which is 50-fold lower than the EC_{50} for αNGF.

αNGF Modulation of Steady-State Binding at 0.5 °C. In order to investigate the effect of αNGF on the steady-state binding of ^{125}I-βNGF over a wide range of βNGF concentration, experiments were performed at a constant, high concentration of αNGF. Incubations were for 100 min, and total binding (in the absence of αNGF), binding in the presence of a large excess (1000-fold) of unlabeled βNGF, and binding in the presence of excess αNGF were assayed in parallel. The residual binding with excess αNGF fit a linear model appropriate for "nonspecific binding" up to the highest ^{125}I-βNGF concentration measured, 2 nM. The linear correlations are highly significant, and the slopes determined from these analyses were 10.3 ± 0.15 fmol/(million cells·nM) with excess

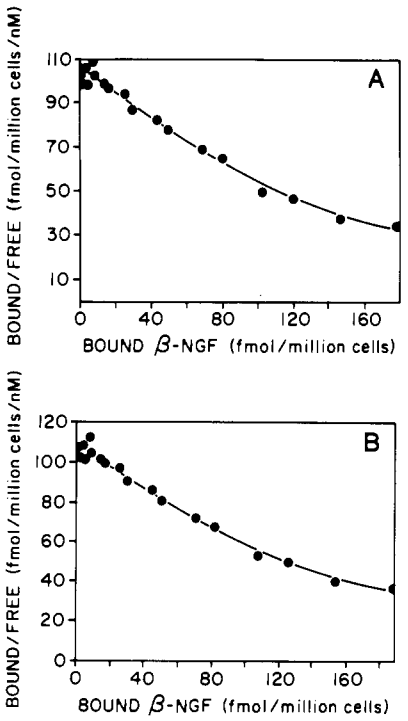

FIGURE 2: Scatchard plot of αNGF or γNGF modulation of ^{125}I-βNGF binding at 0.5 °C. PC12 cells (1.6 million cells/mL) were incubated with competing subunit for 100 min over a range of ^{125}I-βNGF concentrations from 2 pM to 5 nM. Difference data were calculated by subtracting the residual bound ^{125}I-βNGF in the presence of 40 μM αNGF (panel A) or 62 μM γNGF (panel B) from the total bound βNGF in the absence of subunits. The slope of the straight line in the presence of αNGF was 3.42 ± 0.04 fmol/(million cells·nM) and in the presence of γNGF was 0.46 ± 0.02 fmol/(million cells·nM). The solid line in each panel is the theoretical line generated from the best-fit parameters from the LIGAND program and listed in Table I. Root mean square (RMS) error was 3.8% and 2.6% for α and γ difference data, respectively.

unlabeled βNGF and 3.4 ± 0.04 fmol/(million cells·nM) with 40 μM αNGF. These values indicate that the residual binding in the presence a αNGF at 0.5 °C is reduced significantly below that of the nonspecific binding determined experimentally with a large excess of unlabeled βNGF.

The differences between the total binding and binding in the presence of either unlabeled βNGF or unlabeled αNGF were calculated. These data are presented as "difference data" (Figure 2) as we have previously done (Woodruff & Neet, 1982) in analogy with "specific binding" from the difference in the presence of excess unlabeled βNGF. The resulting difference data were analyzed by the LIGAND fitting program (see Materials and Methods), and the binding parameters best explaining the data were for a single, independent binding class (Table I). The difference data are shown plotted on Scatchard coordinates (Figure 2A) in which the solid line represents the computer-fitted parameters. These independent computer fits to each data set suggest that no significant difference (at the 95% confidence level) exists between the data sets for either the apparent K_d or the apparent binding site concentration (Table I). The fit to the αNGF difference data indicates that a significant linear component is present; in contrast, however, the fit to the βNGF difference data shows no linear component (Woodruff & Neet, 1986). Since the βNGF difference data is the same as the specific binding, a linear component was not expected. The linear component found in the αNGF difference data appears to result from the lowered residual binding in the presence of αNGF as compared with the βNGF residual (nonspecific) binding (Figure 2).

Table I: Summary of Steady-State Binding to PC12 Cells[a]

	temperature of binding	
	0 °C	37 °C
Specific ^{125}I-βNGF Binding		
R_0 (sites/cell)	99 000 ± 7600	157 000 ± 20 000
K_d (nM)	1.7 ± 0.06	0.57 ± 0.07
nonspecific binding[b] [sites/(cell·pM)]	6.2 ± 0.1	3.0 ± 0.6
αNGF Difference Data		
R_0 (sites/cell)	82 700 ± 9200	126 000 ± 11 000
K_d (nM)	1.47 ± 0.14	0.47 ± 0.05
additional linear component[c] [sites/(cell·pM)]	7.8 ± 1.5	0.6 ± 3
residual binding[b] [sites/(cell·pM)]	2.1 ± 0.02	21.6 ± 0.6
γNGF Difference Data		
R_0 (sites/cell)	84 000 ± 9000	
K_d (nM)	1.5 ± 0.14	
additional linear component[c] [sites/(cell·pM)]	9 ± 1.5	
residual binding[b] [sites/(cell·pM)]	0.3 ± 0.01	

[a] Parameters and standard errors were obtained from LIGAND analysis of specific ^{125}I-βNGF binding data and of difference data obtained in the presence of either αNGF or γNGF shown in Figures 2 and 3. Sites/cell = 605 × fmol/million cells. [b] Experimental measurement of residual binding in the presence of unlabeled βNGF (1050-fold excess), αNGF (37–40 μM), or γNGF (62 μM). [c] Calculated from the LIGAND analysis and contained in the difference data.

When the data sets were fit simultaneously (not shown), the runs test value indicated that the βNGF difference data were significantly different from the αNGF difference data. This difference most probably resulted from the presence of the linear component in the αNGF difference data and its absence from the βNGF difference curve as described above. The simultaneous fit of the total binding data and the αNGF difference data suggests that the residual binding in the presence of αNGF is minimal, in agreement with the linear regression analysis.

γNGF Modulation of Steady-State Binding at 0.5 °C. Investigation of the effect of γNGF on the steady-state binding of ^{125}I-βNGF at 0.5 °C was also carried out in a fashion analogous to that used for αNGF. Incubations in the presence or absence of 62 μM γNGF for 100 min at 0.5 °C were performed for the whole range of ^{125}I-βNGF concentrations. When the results were plotted directly, the binding that remained in the presence of γNGF was linear up to 5.5 nM ^{125}I-βNGF concentrations (not shown). The slope was 0.46 fmol/(million cells·nM), indicating a very low level of residual binding. These data were analyzed as the difference (total minus remaining binding) and computer fit to binding models. Figure 2B shows the difference data plotted by the method of Scatchard along with the lines generated from the parameters determined from the best computer fit (Table I). The number of sites and the affinity determined from these difference data were not significantly different from the analysis of the total binding curve or the βNGF specific binding. An additional linear component found in the γNGF difference data [15 fmol/(million cells·nM)] gave a close approximation to the linear component determined to be present in the total binding curve [16 fmol/(million cells·nM)] or the βNGF nonspecific binding [10.3 fmol/(million cells·mM)]. These results closely parallel the αNGF findings.

αNGF Modulation of Steady-State Binding at 37 °C. The effect of αNGF on the steady-state binding of ^{125}I-βNGF was also examined at 37 °C. In contrast to the 0.5 °C results, the binding remaining in the presence of 37 μM αNGF at 37 °C

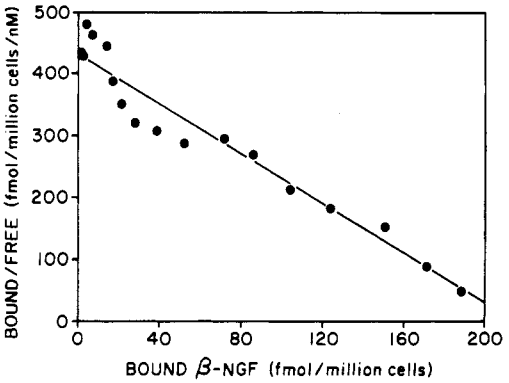

FIGURE 3: Scatchard plot of αNGF modulation of ^{125}I-βNGF binding at 37 °C. PC12 cells (1.43 million/mL) were incubated with competing αNGF for 40 min over a range of ^{125}I-βNGF concentrations from 3.4 pM to 4 nM. Difference data were calculated by subtracting the residual bound ^{125}I-βNGF in the presence of 37 μM αNGF from the total bound βNGF in the absence of α subunit. The slope of the straight line below 0.5 nM ^{125}I-βNGF in the presence of αNGF was 35.8 ± 1.0 fmol/(million cells·nM). The solid line is the theoretical line generated from the best-fit parameters from the LIGAND program and listed in Table I. RMS error was 5.2%.

Table II: βNGF Dissociation Kinetics Induced by αNGF[a]

temperature (°C)	k_{-1} (s^{-1})	$t_{1/2}$	sites/cell	%
	Component 1			
0.5	2.5×10^{-2}	27.7 s	11400	51.5
37	4.9×10^{-2}	14.14 s	9900	71.0
	Component 2			
0.5	1.4×10^{-3}	8.2 min	6400	29.2
37	7.8×10^{-4}	14.8 min	3100	22.0
	Component 3			
0.5	2.8×10^{-6}	69 h	4300	19.3
37	3.1×10^{-6}	62 h	1000	7.0

[a] Parameters were obtained by fitting the data of Figure 4 at two temperatures of dissociation to the expression for a three-component exponential dissociation model. The calculated initial level, just prior to dissociation, of binding to each component (r_i) is also reported, both as the absolute amount and as the percentage of the total initial binding. These values were derived from the analytical model described under Materials and Methods.

was linear only up to about 0.5 nM and then curved downward (data not shown); the slope of 35.8 fmol/(million cells·nM) [21.6 sites/(cell·pM), Table I] below 0.5 nM was also anomalously high. Figure 3 shows the difference data (total binding minus binding experimentally measured in the presence of 37 μM αNGF) plotted as transformed by the method of Scatchard. When these binding difference data were analyzed by the LIGAND program, the model best explaining the experimental results was a single class of sites having an apparent affinity of 0.47 ± 0.05 nM and consisting of 126000 ± 11000 sites/cell (Table I). These results are not significantly different from the specific binding data generated in the presence of an excess (1000-fold) of unlabeled βNGF (Schechter & Bothwell, 1981; Woodruff & Neet, 1986; see also Table I). Similar values were also obtained when the total βNGF binding data were fit by LIGAND prior to correction for nonspecific binding (Woodruff & Neet, 1986). When both the specific ^{125}I-βNGF data and the αNGF difference data were simultaneously computer fit with LIGAND, the result confirmed that this model with a K_d of 0.52 ± 0.05 nM and 150000 ± 13000 sites/cell fit both experimental data sets; neither individual set was significantly different from the simultaneous fit. Despite the agreement between the βNGF and αNGF difference data at 37 °C, the latter must be taken as an underestimate of the true amount of ^{125}I-βNGF specifically

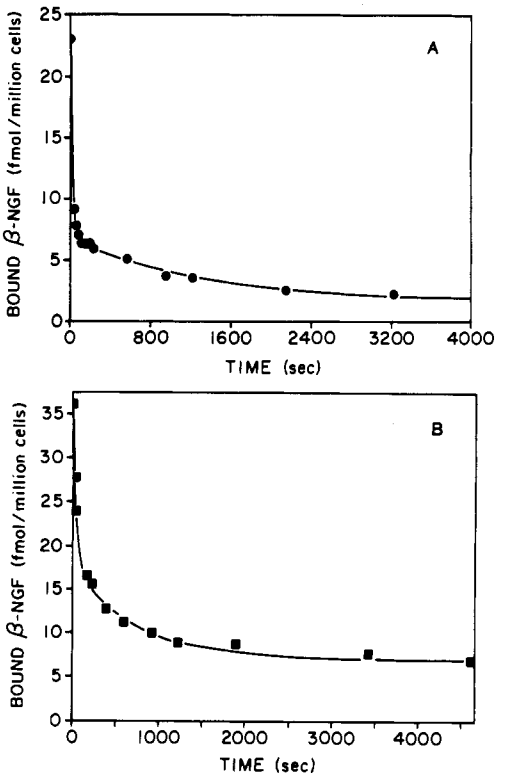

FIGURE 4: Dissociation kinetics of βNGF induced by αNGF at 0.5 or 37 °C. (Panel A) Dissociation at 37 °C. PC12 cells (1.8 million cells/mL) were incubated with 110 pM ^{125}I-βNGF for 30 min at 37 °C, which resulted in a specific binding of 23.1 fmol/million cells. The chase was initiated by adding αNGF to a final concentration of 36.4 μM with a resultant dilution to 1.6 million cells/mL. (Panel B) Dissociation at 0.5 °C. PC12 cells (1.8 million cells/mL) were incubated with 155 mP ^{125}I-βNGF for 30 min at 37 °C, which resulted in a specific binding of 36.7 fmol/million cells. The chase was initiated by cooling the cells for 5 min and then adding precooled αNGF to a final concentration of 36.4 μM with a resultant dilution to 1.6 million cells/mL. The fitting procedure to the dissociation rate equation has been described (Woodruff & Neet, 1986) and was extended here to include three exponential terms (see Materials and Methods). At both temperatures the residuals were randomly distributed above and below the solid line shown that is predicted by the fitted parameters (Table II). In panel A there are five residuals below and eight above and five runs; the minimum number of runs at the 95% confidence limit is three. In panel B there are six residuals below and six above and eight runs; the minimum number of runs at the 95% confidence limit is four. The parameters may not be unique, however, since other, untried combinations may also provide adequate fits.

competed for by αNGF because of the anomalous "residual binding" (see Discussion).

αNGF Modulation of βNGF Dissociation Kinetics. Following an incubation period of 30 min at 37 °C with ^{125}I-βNGF, PC12 cells were exposed to a concentration of αNGF that produced a maximal effect in the previous steady-state experiments. The time course of ^{125}I-βNGF dissociation was then followed at either 37 °C or at 0.5 °C.

The same analytical procedures used for βNGF-induced dissociation experiments (see Materials and Methods) showed that neither a monoexponential nor a biphasic model could explain the 37 °C data adequately (not shown). A model comprised of three dissociation components, however, fit the data quite well (Figure 4 and Table II) as determined by a runs test, which indicated a random distribution (above the 95% confidence level) of residuals (Mannervik, 1982). The analysis of the data for the 37 °C αNGF chase indicates that the first component has a half-time that is not significantly different from that seen with βNGF initiation of dissociation at 37 °C (Woodruff & Neet, 1986; see also Table II). The

second component was also numerically very similar to the βNGF-initiated slow component at 37 °C. The third component, however, dissociates very slowly ($t_{1/2}$ = 62.1 h) and represents about 7% of the initially bound ^{125}I-βNGF for this experiment. This third component at 37 °C does not correspond to any βNGF-initiated component and may be a result of the incomplete binding of free βNGF by αNGF under these conditions.

Whereas lowering the temperature to 0.5 °C during the βNGF-initiated dissociation markedly affects the slowly dissociating component and only slightly retards the rapidly dissociating component (Schechter & Bothwell, 1981; Landreth & Shooter, 1980; Woodruff & Neet, 1986), the effect observed when αNGF is used somewhat different (Figure 4B and Table II). Again, a three-component model was required with the αNGF-initiated cold chase data, since simpler models were not able to fit the data with any statistical significance. Decreasing the dissociation temperature with added αNGF appeared to increase the percent of slowest component, which had a dissociation rate constant very similar to that with βNGF chase at 0.5 °C (Woodruff & Neet, (1986).

αNGF Cold Chase Stable ^{125}I-βNGF Binding. In order to correlate the kinetic components found with the βNGF dissociation experiments, the dependence on ^{125}I-βNGF concentration was examined in experiments analogous to the βNGF cold chase experiments (Schechter & Bothwell, 1981; Landreth & Shooter, 1980; Chandler & Herschman, 1983; Woodruff & Neet, 1986). Following an ^{125}I-βNGF association incubation at 37 °C, the cells were cooled, and a chase concentration of αNGF was added at 0.5 °C. Exactly 20 min later triplicate samples were assayed for ^{125}I-βNGF binding. At this point in the complex dissociation curve virtually all of the rapidly dissociating component (component 1) has dissociated. Most of the second component (component 2) has also dissociated (81.4% of that which was initially bound). Only a small percentage (0.3%) of the third component (component 3) has dissociated. Of the ^{125}I-βNGF remaining bound, 22% is bound to component 2 and 78% is bound to component 3. Although this experiment was unable to cleanly resolve the dissociating components, the fact that component 1 was more readily resolved suggested that the results from the αNGF cold chase stable βNGF binding would provide significant information in comparison with the βNGF cold chase experiments. These results are plotted directly (Figure 5A) (free ^{125}I-βNGF concentration vs. bound ^{125}I-βNGF) and in Scatchard coordinates (Figure 5B). When evaluated by the LIGAND program, the only model providing an adequate fit to the curve was a single class of positively interacting sites with an additional linear component. The parameters yielding the best fit to this complex curve suggest that the 4000 sites/cell have a K_d of 57 pM and interact with significant positive cooperativity (B = 5.9). A linear, nonsaturated component was also present comprising 25 fmol/(million cells·nM) of the total binding.

γNGF Cold Chase Experiments. Since the steady-state equilibrium results found with γNGF at 0.5 °C were qualitatively similar to the αNGF results, the more complex experiments examining the cold γNGF chase stable ^{125}I-βNGF binding were undertaken to attempt to discriminate between the effects of these two 7S oligomer subunits. A saturating concentration of γNGF was used to "chase" bound, labeled βNGF from PC12 cells at 0.5 °C. Initial association of ^{125}I-βNGF was allowed to proceed at 37 °C for 30 min prior to cooling cells and addition of the chase γNGF. Binding assays in triplicate were carried out after exactly 20 min of incubation at 0.5 °C, and the resulting stable binding was plotted vs. the free ^{125}I-βNGF concentration. When these data were fit to various models, the model providing the best fit was again a single class of positively interacting sites along with a linear component. None of the more complex models would fit the data and often would reduce to the single-class model. The binding data, plotted on Scatchard coordinates (Figure 6), demonstrate the qualitative similarity to the binding sites that are stable to an αNGF or βNGF chase. The quantitative effect of γNGF was also very similar to the effect of αNGF (Table III). Therefore, both α and γ subunits of 7S NGF as well as βNGF itself act as chase proteins and define a similar class of PC12 sites with complex binding properties. All three NGF subunits also fail to dissociate a component, not saturated with ^{125}I-βNGF below 400 pM, of about the same size.

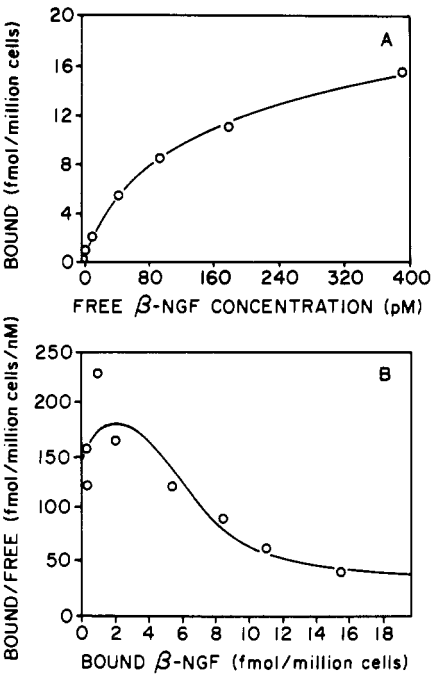

FIGURE 5: αNGF-initiated cold chase stable binding. Various concentrations of ^{125}I-βNGF were allowed to bind to PC12 cells (1.5 million cells/mL) for 30 min at 37 °C and then cooled to 0.5 °C and chased for 20 min with addition of 45 μM αNGF (final concentration of 1.01 million cells/mL). The resulting bound ^{125}I-βNGF is presented as the binding isotherm in panel A and in the form of a Scatchard plot in panel B. In each panel the solid line is the theoretical line drawn to the parameters determined by the LIGAND program and presented in Table III.

Table III: Summary of Cold Chase Stable Binding Parameters[a]

	chase protein		
	βNGF[b]	αNGF[c]	γNGF[d]
K_d (pM)	48 ± 5	57.0 ± 18	48.0 ± 9
R_0 (sites/cell)	2500 ± 240	4000 ± 1800	3500 ± 890
cooperativity parameter B	10.3 ± 3.6	5.9 ± 7.7	5.6 ± 4.1
second dissociation constant[e] (pM)	4.7	9.7	8.6
R_0/K_d, nonsaturated[f] [sites/(cell·pM)]	16 ± 1	15 ± 7	20 ± 4

[a] Parameters were obtained from LIGAND analysis of binding data for ^{125}I-βNGF remaining after displacement by unlabeled subunit, as indicated, for 30 min at 0.5 °C with no nonspecific subtraction. Sites/cell = 605 × fmol/million cells. [b] βNGF from a single experiment done at the same time as α- and γNGF. These numbers differ slightly from the combined results (Woodruff & Neet, 1986). [c] αNGF from Figure 5. [d] γNGF from Figure 6. [e] Calculated as K_d/B. [f] Includes contributions from both nonspecific and nonsaturated components at the concentrations of ^{125}I-βNGF below 400 pM used here.

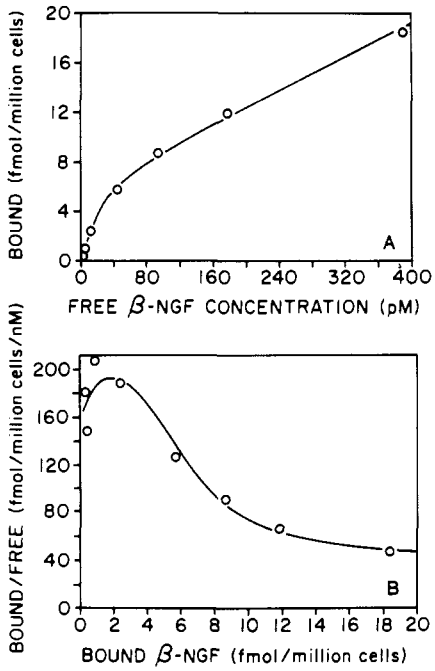

FIGURE 6: γNGF-initiated cold chase stable binding. Various concentrations of ^{125}I-βNGF were allowed to bind to PC12 cells (1.5 million cells/mL) for 30 min at 37 °C and then were cooled to 0.5 °C and chased for 20 min with the addition of 62 μM γNGF (final concentration of 1.01 million cells/mL). The resulting bound ^{125}I-βNGF is presented as the binding isotherm in panel A and in the form of a Scatchard plot in panel B. In each panel the solid line is the theoretical line drawn to the parameters determined by the LIGAND program and presented in Table III.

DISCUSSION

The work presented here describes the effects of the other protein components of the oligomeric 7S NGF on the βNGF subunit binding to PC12 cells. αNGF and γNGF act in a similar fashion to prevent the binding of labeled βNGF to PC12 cells, albeit at different concentrations. The binding of βNGF to the receptor [shown in simplified form in eq 1a; cf. Woodruff and Neet (1986)] could be inhibited through either (i) direct competition for cellular binding sites by subunit binding to receptor (eq 1b and 1c) or (ii) formation of αβ and βγ complexes in solution that themselves are incapable of binding to receptor (eq 1d and 1e). No ternary complexes

$$R + \beta \rightleftharpoons \beta R \quad (K_d^\beta) \tag{1a}$$

$$R + \alpha \rightleftharpoons \alpha R \quad (K_d^\alpha) \tag{1b}$$

$$R + \gamma \rightleftharpoons \gamma R \quad (K_d^\gamma) \tag{1c}$$

$$\beta + \alpha \rightleftharpoons \alpha\beta \quad (K_d^\alpha) \tag{1d}$$

$$\beta + \gamma \rightleftharpoons \beta\gamma \quad (K_d^\gamma) \tag{1e}$$

would form in either case. In the first mechanism, eq 1b and 1c, K_d^α or K_d^γ would represent the dissociation constant of α or γ from the receptor; in the latter mechanism, eq 1d and 1e, the constants have the same value but now would represent the dissociation constants of the αβ and βγ solution complexes to individual subunits in the absence of receptor. For either mode of displacement by αNGF or γNGF the mathematical form is the same (eq 2), and the K_d characterizing the interaction can be calculated. Since the concentration of

$$K_d = \frac{EC_{50}}{1 + [\beta]/K_d^\beta} \tag{2}$$

^{125}I-βNGF is known (1 nM) and the K_d^β describing the interaction of labeled βNGF with those sites has been determined directly to be 0.52 nM (Woodruff & Neet, 1986), then the K_d^α, calculated from an EC_{50}^α of 9–10 μM (Figure 1) would be 3.3–3.5 μM. Similarly, for γNGF, an EC_{50}^γ of 200 nM (Figure 1) would generate a K_d^γ of about 67 nM. The experiments to conclusively rule out direct subunit binding at receptor sites are not experimentally feasible due to the high level of nonspecific binding at the required high concentration. Assuming the same concentration of binding sites (10^5 sites/cell) and a level of nonspecific binding in the range found for ^{125}I-βNGF binding to PC12 cells [11 sites/(cell·pM)] and also for ^{125}I-αNGF binding to ganglionic cells (Woodruff & Neet, 1982), the specific binding would not be distinguishable from the nonspecific. At 3 μM labeled αNGF the nonspecific binding would be 99.8% of the total; at 0.1 μM labeled γNGF the nonspecific binding would still be 95%. Thus, determination of direct binding of αNGF or γNGF to PC12 receptors as the mechanism of inhibition is not technically possible. However, since both αNGF and γNGF effects correspond well with their abilities to form complexes with βNGF in solution, a more attractive explanation is apparent. The K_d for the βγ interaction is 100–500 nM (Woodruff, 1983; Almon & Varon, 1978; Nichols & Shooter, 1983; Bothwell & Shooter, 1978), while for αNGF the K_d for αβ complex interaction is 5–6 μM (Woodruff, 1983; Almon & Varon, 1978). Each of these constants is close to the value calculated above from the receptor competition studies, and we conclude that the binary complexes formed in solution (eq 1d and 1e) do not bind receptor. The kinetic experiments of Harris-Warrick et al. (1980) and Almon and Varon (1978) suggest indirectly that the rate of association of αNGF with βNGF would not be rate-limiting in the chase experiments performed here. Therefore, the chase experiments are also compatible with a model of complex formation in solution. Thus these experiments, taken with the model for the binding of ^{125}I-βNGF developed in the preceding paper (Woodruff & Neet, 1986), would suggest a scheme in which αβ or βγ complexes do not bind to any form of the PC12 receptor. In contrast, with the ganglionic system the αβ complex was able to bind to the lower affinity ganglionic receptor, resulting in the experimental inability of αNGF to cause complete competition with dorsal root neurons (Woodruff & Neet, 1982).

The fits to the 0.5 °C steady-state data when binding in the presence of either αNGF or γNGF is subtracted from the total ^{125}I-βNGF binding demonstrate a paradoxical additional linear component in the analysis of these difference data. When the specific difference data were derived from unlabeled βNGF coincubation, however, the computer fit does not resolve any linear component (Woodruff & Neet, 1986). This linear component found with αNGF and γNGF displacement but not with unlabeled βNGF displacement suggests that the formation of ^{125}I-βNGF complexes with the other NGF subunits produces a species with different nonspecific binding characteristics. The difference in charge characteristics between βNGF, which is very basic, and either the αβ or βγ complex, each of which is more neutral, may also result in a decrease in the other cellular sites with which the complexes are able to interact. This blocked, nonspecific binding would therefore be evident as a linear component in the binding difference data analysis.

The cold chase stable binding experiments further define the properties of the PC12 receptor classes. The effect of either αNGF or γNGF under these chase conditions mimics the effect of βNGF itself, defining a class of sites that exhibit positive cooperativity and high affinity and also possess a

significant linear component. This linear "nonsaturated" component is quantitatively similar whether βNGF is used to initiate the chase or either αNGF or γNGF is used and was previously interpreted to be the result of the relatively low ^{125}I-βNGF concentrations utilized (Woodruff & Neet, 1986). This linear, nonsaturated component in the cold chase results should not be confused with the 0.5 °C steady-state results in which a linear component was observed in the αNGF and γNGF difference data (but not in the βNGF difference, or specific, binding data) and was attributed to the displacement of a portion of the true nonspecific component (see above).

From the displacement curve with αNGF (Figure 1), a concentration of 37 μM αNGF is calculated to displace 77–81% of the ^{125}I-βNGF at 37 °C. This figure is consistent with a dissociation constant of about 2 μM for the αβ complex, in the range of that observed (Woodruff, 1983; Almon & Varon, 1978). The lack of complete complexation of the ^{125}I-βNGF accounts for the high residual binding and lack of linearity above 0.5 nM ^{125}I-βNGF observed with αNGF competition at 37° C (Figure 3 and Table I). Nevertheless, the difference data observed at 37 °C still represents a good approximation of the binding characteristics of the ^{125}I-βNGF that did not bind due to the competition between αNGF and the cellular receptor. The determined parameter values would be off by the same 20% since this error would be a constant proportion throughout the ^{125}I-βNGF range. The observation that the αNGF competition in the cold leads to a much lower residual component (2.1 sites/(cell·pM), Table I) suggests that the K_d for αβ dissociation is 3–5-fold lower at 0.5 °C than at 37 °C, thus causing a much more effective competition. Unfortunately, insufficient data in the literature exists to corroborate this prediction. The cold chase stable receptor binding with αNGF displacement data (Figure 5) should be satisfactory since the complex can readily form at 0.5 °C with this protocol. Similarly, the γNGF competition studies are straightforward since the K_d, about 0.4 μM, is much lower than the γNGF concentration used (62 μM) and resulted in 98% reduction in βNGF binding (Figure 1). A detailed, quantitative interpretation of the αNGF-initiated, kinetic dissociation curves at 37 °C (Figure 4 and Table II) would not be appropriate because of the lack of complete αβ complex formation attainable under these experimental conditions.

The addition of either αNGF, γNGF, or unlabeled βNGF has basically the same effect on the kinetics of ^{125}I-βNGF dissociation (Table II) or on the cold chase stable bound ^{125}I-βNGF (Table III). This observation has relevance to the discussion of positive cooperativity with PC12 cells and to the discussion of negative cooperativity and unstirred layer effects with NGF receptors, done primarily with neurons (Frazier et al., 1974; Sutter et al., 1979; Olender & Stach, 1980; Riopelle et al., 1980; Tait et al., 1981). The enhanced rate of dissociation seen with a chase experiment compared to dilution (Yankner & Shooter, 1979; Neet et al., 1984) cannot be due to negative cooperativity in the PC12 system since addition of αNGF or γNGF would not fill empty βNGF receptors to enhance dissociation, as proposed by the negative cooperativity model. Since α-, β-, and γNGF are all about the same size, they should have the same accessibility to an "unstirred layer", thus accounting for the similarity of dissociation properties of labeled βNGF and validating these rates as the best representation of the dissociation. Greater than 90% of the ^{125}I-βNGF dissociates in a multiphasic manner in the presence of αNGF at 37 °C (Figure 4), supporting the interpretation that this process is dissociation from a heterogeneous cell surface receptor.

The functional significance of the two or three classes of cellular receptors with different affinities or, in the case of the PC12 cells, different kinetic properties has not yet been fully interpreted. The wide range of effects observed in response to βNGF suggests that they are not all necessarily mediated through the same class of receptors. The survival and neurite outgrowth stimulating properties of βNGF apparently correlate with occupancy of the high-affinity (site I) receptors on dorsal root ganglionic and sympathetic cells (Greene & Shooter, 1980; Sutter et al., 1979). This conclusion is primarily based on the similarity in dose–response curves and the low receptor occupancy if the low-affinity receptors were mediating this response. Futher, the work by Stach and Wagner (1982) suggests that the site II receptors of neurons are not mediating this response in conjunction with the site I receptors since cells from a different strain of chicken, which possess 2.5-fold fewer site II receptors, show no change in the dose–response curve with βNGF. Baribault and Neet (1985) have recently reported that the relationship may be more complex in the PC12 system, on the basis of inhibition of processing of the glycoprotein receptor. Kedes et al. (1982) have suggested that both receptor classes are involved in the stimulation of α-aminoisobutyric acid uptake in PC12 cells.

The inhibition of α and γ subunits reported here with all receptor forms of PC12 cells and previously found only with high-affinity receptors of dorsal root neurons (Harris-Warrick et al., 1980; Woodruff & Neet, 1982) strongly supports the conclusion that distinctive differences exist between PC12 cells and neurons with regard to βNGF receptor interactions. Although the details behind the apparent positively cooperative steady-state isotherms (Figures 5 and 6; Woodruff & Neet, 1986) with PC12 cells are unknown, this observation may also suggest a distinguishing feature between PC12 and ganglionic cells. Whether these distinctions are due to a qualitative, molecular difference or are the result of quantitative differences cannot yet be answered.

The evidence presented here indicates that both the α subunit and the γ subunit competitively block the binding of αNGF to the PC12 receptor. However, αNGF and γNGF cannot both be binding to the same site on the βNGF dimer since the 7S NGF oligomer is an $\alpha_2\beta\gamma_2$ complex. Either a conformational change must occur in βNGF upon binding of αNGF and/or γNGF (Bothwell & Shooter, 1978; Rao & Neet, 1984) or the sites of α and γ binding are sufficiently close that these subunits of 26 000 daltons each sterically interfere with the β-dimer (26 000-Da) receptor site. Although the local concentrations of the NGF subunits at the site of action in vivo are completely unknown, the high K_d for either the βγ or αβ complex relative to the biological EC_{50} or the K_d for βNGF receptor binding suggests that modulation of the physiological function of βNGF is not likely. However, the 7S NGF equilibrium with its dissociated forms is clearly complex (Bothwell & Shooter, 1978; Palmer & Neet, 1980a,b; Nichols & Shooter, 1983), and the addition of αNGF to a βγ equilibrium is known to have dramatic effects (Bothwell & Shooter, 1978; Rao & Neet, 1984; Silverman & Bradshaw, 1982). The utilization of the α and γ subunits may prove useful in mapping the "active site" and conformational requirements for biological activity of βNGF.

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