β Nerve Growth Factor Binding to PC12 Cells. Association Kinetics and Cooperative Interactions†

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ABSTRACT: The association kinetics of ^{125}I β nerve growth factor (NGF) binding to the PC12 clonal cell line have been examined in detail at 0.5 and 37 °C. These data were examined by utilizing a reversible second-order integrated rate equation, and the results were not consistent with a simple bimolecular process. Two association rates were required to explain the results adequately. At 37 °C, the faster component was estimated to have a second-order association rate constant of 1.4×10^7 M^{-1} s^{-1}, while the rate constant for the slower component (3.8×10^6 M^{-1} s^{-1}) was about 4-fold lower. As shown by others, the temperature dependence of the dissociation kinetics indicated that while the rapidly dissociating component was only slightly slowed by lowering the chase temperature to 0.5 °C, the second component was slowed by about 270-fold, from 8×10^{-4} s^{-1} to 3×10^{-6} s^{-1}. The binding data that describe the slowly dissociating component were obtained by utilizing this differential temperature dependence and revealed a concave downward Scatchard plot. The binding parameters determined from computer analysis using a nonlinear fitting program (LIGAND) suggest that this component consists of (a) an interacting class of about 4000 sites/cell that have a first stoichiometric steady-state dissociation constant of 65 pM and a second stoichiometric interaction constant of 16 pM, indicative of positively cooperative interactions, and (b) a class of sites consistent with a ratio of sites/K_d of about 11.1 sites/(cell·pM). The steady-state binding results at 37 °C indicated only one class of binding sites (155 000 ± 18 000 sites/cell) that had an apparent K_d of 0.52 ± 0.03 nM. One class of sites was also observed at 0.5 °C, and the receptor concentration was found to be reduced (99 000 ± 7600 sites/cell) while the K_d was increased (1.7 ± 0.14 nM). A significant level of positively cooperative interactions was observed frequently at 37 °C that was not due to a failure to reach steady-state conditions, internalization, or degradation. Since cooperativity of binding was never observed at 0.5 °C, a membrane event may be involved. Determination of the contribution of the different classes of NGF receptors found on PC12 cells to the biological actions of NGF requires a clear understanding of their kinetic properties and their relationship to each other. The studies presented here indicate that their interactions are more complex than previously described.

The steady-state binding of βNGF[1] to cell surface receptors on PC12 cells has been investigated by a number of groups since the establishment of this clonal cell line by Greene and Tischler (1976). The investigations have produced differing results but have collectively demonstrated the underlying complexity involved in the βNGF–PC12 cell interactions. The steady-state binding was found by Herrup and Thoenen (1979) to differ significantly from the reported results of βNGF binding to dissociated cells from dorsal root ganglia (Sutter et al., 1979). Instead of finding distinct classes of receptors differing in affinity, they reported one class of sites (58 000 sites/cell) with an apparent K_d of 2.9 nM at 0.5 °C. Herrup and Thoenen did not show any kinetic data from experiments at either room temperature or 37 °C but suggested that the interactions were more complex than "simple association–dissociation kinetics". A more recent paper (Chandler et al., 1984) has reported a similar K_d (3.2 nM) but a larger number of measurable receptors at low temperature, 180 000 sites/cell. Schechter and Bothwell (1981) reported on steady-state binding studies at 37 °C, which they interpreted as indicative of a single class of receptors, 40 000–80 000 sites/cell, with higher affinity (0.2 nM). Lyons et al. (1983), however, reported biphasic binding at room temperature to solubilized receptors from PC12 cells with K_d values of about 38 pM and 5 nM. Bernd and Greene (1984) also reported values of 0.33 nM and 3.8 nM at 37 °C for cell surface receptors after correction for acid-stable NGF. Yankner and Shooter (1979) found a nonlinear Scatchard plot for the binding of βNGF at 37 °C to a "nuclear fraction" of PC12 cells, indicating two classes of sites with K_d values of 80 pM and 9 nM. A summary of steady-state binding to PC12 cells has been made (Vale & Shooter, 1985).

The kinetics of the dissociation of βNGF to PC12 cells are also complex. At 37 °C, in a similar fashion as that observed with dorsal root ganglionic cells (Sutter et al., 1979), the dissociation of bound ^{125}I-βNGF, induced by the addition of a large excess of unlabeled βNGF, is not a single exponential. Landreth and Shooter (1980) found that two dissociation components could be discerned, one with a $t_{1/2}$ of 30 s ($k_{-1} = 2 \times 10^{-2}$ s^{-1}) and the other with a $t_{1/2}$ of 20 min ($k_{-2} = 4 \times 10^{-4}$ s^{-1}). If the dissociation was carried out at 0.5 °C, however, the dissociation of the slower component was almost eliminated. In contrast, the effect on the rapidly dissociating

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[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; PC12, pheochromocytoma 12 cell line; BSA, bovine serum albumin; TCA, trichloroacetic acid; RMS, root mean square; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate.

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component was relatively small. Landreth and Shooter (1980) hypothesized that the slowly dissociated component represented the high-affinity sites that they observed in steady-state binding. By use of the differential change in dissociation constants when the temperature is lowered, the time course and properties of this cold chase stable component were investigated. A conversion of low-affinity to high-affinity receptors was proposed (Landreth & Shooter, 1980).

Schechter and Bothwell (1981) have also found two kinetic components of the dissociation of ^{125}I-βNGF bound to PC12 cells. In close agreement with Landreth and Shooter (1980), the dissociation rate constants at 37 °C were determined to be 2.3×10^{-2} s^{-1} for the rapidly dissociating sites and 5.8×10^{-4} s^{-1} for the slowly dissociating sites. When the chase temperature was lowered to 0.5 °C, their results were similar to those reported by Landreth and Shooter (1980). Schechter and Bothwell (1981) concluded that two preexisting classes of sites were present but needed to be reconciled with their steady-state experiments, which suggested one class of sites. These investigators suggested that different association constants for the two classes of sites could be balancing the large difference in dissociation rates, thereby resulting in steady-state equilibrium constants not sufficiently different to be resolved experimentally (Schechter & Bothwell, 1981). However, this hypothesis has not been rigorously tested. Schechter and Bothwell (1981) also presented steady-state binding data in which the cold chase stable component ("slow" receptor) was determined experimentally and plotted by the method of Scatchard. Although their results suggested nonlinearity in binding, they utilized a simple, homogeneous, noninteracting receptor model with which to analyze the Scatchard data. Since they found an apparent equilibrium constant of 200 pM, the same as they found for the total specific binding at 37 °C, they suggested that the apparent affinities are the same for the two classes of sites.

Discrepancies between reported binding data could be due to differences in experimental protocols, to limited types of available data, or to differences in the PC12 tumor cell line arising from its known instability. In this report we describe the association kinetics for the binding of βNGF to PC12 cells and examine the binding interactions among the slowly dissociating class of sites. We have also analyzed the steady-state binding properties of the βNGF receptor by means of a nonlinear computer program, LIGAND, for receptor binding (Feldman et al., 1972; Munson, 1983) and developed a steady-state binding model. A thorough investigation of the kinetics and steady-state binding of βNGF to the cellular receptor was necessitated by our studies, reported in the following paper (Woodruff & Neet, 1986), on the modulation of βNGF binding to PC12 cells by the α and γ subunits of 7S oligomeric NGF.

Materials and Methods

Buffers. 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; the ionic strength was 0.05, and the buffer contained a total of 27.1 mM phosphate anion. Acetate buffer contained 0.05 M sodium acetate and 0.15 M sodium chloride at pH 4.0. Ethanolamine elution buffer contained 0.24 M ethanolamine (ACS spectral grade) and 2.0 M NaCl, pH 10.6. 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 manufacturer's instructions. PBS/BSA solution was PBS made to contain 1 mg/mL BSA (Pentex crystalline); the pH was readjusted to 7.4 and the solution filtered through a 0.22-μm membrane filter to clarify and sterilize.

DMEM (Dulbecco's modified Eagle's medium) was obtained from GIBCO either as 1× liquid or as powder and prepared according to the manufacturer's instructions. 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).

Preparation of NGF. Isolation of 7S NGF was carried out by the method of Stach et al. (1977) with some modifications. Swiss Webster random bred albino male mice (60 days old) were obtained from Charles River Breeders. Twenty to twenty-five grams of submaxillary gland material was routinely obtained from 100 mice and either used directly or stored frozen at −80 °C. The dissociated subunits were isolated on a CM-cellulose column (Whatman CM-52) (Smith et al., 1969), and the concentration was determined by using the known extinction coefficients and molecular weights (Smith et al., 1969; Smith, 1969). The subunits were periodically examined for purity and molecular characteristics by tube gel isoelectric focusing (IEF) as described elsewhere (Woodruff, 1983).

βNGF Bioassay. Biological assays were performed with the PC12 clonal cell line (Greene et al., 1976). These were carried out as described by Greene (1977) with some modification for priming and freezing (Black & Greene, 1982). Response of cells was always 70–80% or better with a half-maximal activity at about 25 pM βNGF.

βNGF Iodination. The protocol for the βNGF iodination reaction was the same as that of Sutter et al. (1979) except that we used reduced concentrations of hydrogen peroxide, multiply divided aliquots of hydrogen peroxide, and a substantially reduced time for the reaction (Woodruff, 1983). The standard conditions employed 50 μg of βNGF, 5.56×10^{-2} units of lactoperoxidase (Calbiochem), and 3 mCi of $^{125}I_2$ (Amersham, carrier free) in a final volume of 60 μL of 0.1 M phosphate buffer, pH 7.4. The reaction was initiated by addition of 10 μL of freshly diluted hydrogen peroxide [(5.88×10^{-4})%] to the reaction tube and mixed by pipetting. Following this, at intervals of 2 min, additional 10-μL aliquots of hydrogen peroxide were mixed into the reaction until a total of 40 μL had been added (6 min). At 10 min after the first hydrogen peroxide addition, the reaction was stopped by adding 200 μL of ice-cold 0.4% acetic acid.

Subsequent workup and calculation of specific activity have been described (Sutter et al., 1979; Woodruff, 1983). Specific radioactivities ranged from 50 to 100 cpm/pg of NGF and better than 95% TCA precipitability.

The iodination of βNGF was evaluated in several ways. Characteristics of the biological activity were periodically checked, and the results suggest that the preparations were not heavily damaged. A few preparations of ^{125}I-βNGF were examined by gel permeation chromatography on a cross-linked agarose gel (AcA 44), by isoelectric focusing, or by SDS gel electrophoresis, with no indication of large amounts of nonnative molecules. Precipitation by cold TCA was assayed in triplicate in 500-μL volumes.

γ counting was done in a scintillation counter (Packard Model 5360 Modumatic VI) with an efficiency of 50–70% and a background of 70–150 cpm. Efficiency of counting was monitored with each run by comparison to a $^{129}I_2$ standard or the peak ratio method and used to convert counts per minute

to disintegrations per minute. Raw counting data were directly punched into paper tape and input into data files that were stored on magnetic media, thus eliminating transcription and calculation errors.

Cells. PC12 cells were grown in complete DMEM as described (Greene, 1977; Woodruff, 1983). Cells were washed and then harvested for experiments by gentle trituration and further washed by centrifugation and resuspension in PBS/BSA buffer. Trypan blue excluding cells usually constituted from 80% to 95% of the cells. The cell concentration was adjusted to give a final density of about 1–2 million cells/mL after counting in a hemocytometer. Two separate PC12 cell cultures were obtained and compared in this work, one that had been used by Landreth and Shooter (1980) and one used by Schecter and Bothwell (1981).

Binding Assay and Analysis. The binding assay followed the one-step sucrose sedimentation protocol described by Sutter et al. (1979) and Vale and Shooter (1985) with minor modifications. At 37 °C a steady-state is reached in less than 24 min for a very low concentration of ^{125}I-βNGF (40 pM) and less than 10 min for a concentration 4-fold higher (170 pM), but to ensure that steady state was reached under all experimental conditions, samples were routinely incubated for 40–45 min. In order to establish that this choice of incubation time was short enough to minimize degradation, radiolabeled βNGF degradation was examined under a variety of conditions by trichloroacetic acid precipitation. TCA has been shown to correlate reasonably well with bioassays or immunoassays for measurement of intact βNGF (Layer & Shooter, 1983). Nonspecific binding was either experimentally evaluated by parallel incubations with a large excess (1000-fold) of unlabeled βNGF or included as a fitted parameter in the binding equation. Recovery of radiolabeled βNGF was routinely measured and was greater than 95% for all binding and degradation experiments.

The means and standard deviations of the triplicate pellets and triplicate supernatants were calculated separately for the total and nonspecific samples. The free ^{125}I-βNGF concentration was calculated from the supernatant portions by taking into account the specific radioactivity of the ^{125}I-βNGF preparation at the time of counting and correcting for the TCA precipitability determined on the day of the experiment. The bound ^{125}I-βNGF was determined from the pellet triplicates in a similar fashion, converted into femtomoles of ^{125}I-βNGF bound, and (by using a molecular weight of 26 000) normalized to one million cells by using the mean and standard deviation of the trypan blue excluding cell counts. Error analysis was performed as described by Bevington (1969). All calculations were performed directly on the raw data without the data having been transcribed by hand. Initial binding data analysis, error analysis, and statistical analysis were carried out on a North Star Horizon Z80 (Zilog) based microcomputer. All programs were written in Fortran 80 (Microsoft) and required the use of an overlay linking loader (Phoenix Software PLINK II).

Curve Fitting. Further analysis of binding data utilized the LIGAND program (Munson, 1983) provided by Dr. Rodbard and modified to run on a DEC System 20. The Marquardt–Levenberg modification of the Newton–Gauss nonlinear regression algorithm is used to find the simultaneous solution of the resulting set of nonlinear equations by the Newton–Raphson technique. The LIGAND program fits the binding data without any mathematical transformation, according to an "$n \times m$" binding model developed by H. A. Feldman (Feldman et al., 1972) with the ability to include cooperativity. The equation (eq 1) in the LIGAND program was originally derived for equilibrium binding but has the same mathematical form as steady-state models (Wiley & Cunningham, 1981; Beck & Goren, 1983) utilized here. The inclusion of the nonspecific binding constant allows analysis of total binding data for which no experimental correction for nonspecific binding was made (cf. Figure 6). The correction factor C allows combination and normalization of various experiments that only differ in receptor concentration (Munson, 1983), thus eliminating one source of error and allowing better analysis (cf. Table I).

A variance model for the bound label that is a linear increase in percentage was reported (Munson & Rodbard, 1979) to be appropriate for protein binding experiments and was experimentally validated for the βNGF binding performed here. The variance for a number of binding experiments was analyzed as a function of the bound label and fit by a separate nonlinear gradient search fitting program. The resulting variance model was used for binding isotherm analysis by the LIGAND program, and the results were compared to the fits by using the variance model suggested (Munson, 1983; Munson & Rodbard, 1979). The binding model determined to give the best fit was the same, and the errors assigned to the fitting parameters, as well as the parameters themselves, were very close to those determined with the experimentally derived variance model. For consistency, the variance model suggested by Rodbard was used throughout, i.e., that in which the variance is a constant percentage of the experimentally determined bound ligand. In order to visually evaluate the fitted parameters and to suggest further models to test, the parameters from all fits were used to produce a simulated binding curve according to eq 1

$$r = C\left[\frac{R_1(K_1\beta + B_1K_1^2\beta^2)}{1 + 2K_1\beta + B_1K_1^2\beta^2} + \frac{R_2(K_2\beta + B_2K_2^2\beta^2)}{1 + 2K_2\beta + B_2K_2^2\beta^2} + (\mathrm{NS})\beta\right] \quad (1)$$

or transformed to the Scatchard plot format. In eq 1, r = bound ^{125}I-βNGF, C = a correction factor for different cellular preparations, NS = the nonspecific binding constant, R_1 = total receptor concentration for site 1, R_2 = total receptor concentration for site 2, K_1 = equilibrium association constant for site 1, K_2 = equilibrium association constant for site 2, β = free ^{125}I-βNGF concentration, B_1 = cooperativity parameter for site 1, and B_2 = cooperativity parameter for site 2. The curve was drawn, along with the original data, by means of a Fortran plotting program, North Star Horizon computer, and Hewlet-Packard (Model 7225A) digital plotter. Each of the first two terms in eq 1 has the form (eq 1A) of a bivalent Adair binding model (eq 1B); i.e., binding of the first ligand molecule to a dimeric receptor occurs with an affinity K, followed by a second ligand at the second site on the dimer with an affinity BK. The cooperativity parameter is simply the ratio between these affinities, B, and represents the change in affinity induced by the first ligand binding. For example, a value of B equal to 4 would correspond to a Hill coefficient of 1.9 (Neet, 1980) (see also Figure 7).

$$v = \frac{R(K\beta + BK^2\beta^2)}{1 + 2K\beta + BK^2\beta^2} \quad (1A)$$

$$\mathrm{R_2} + 2\beta \underset{}{\overset{K}{\rightleftharpoons}} \beta\mathrm{R_2} + \beta \overset{BK}{\rightleftharpoons} \beta_2\mathrm{R_2} \quad (1B)$$

Association Kinetics. The kinetics of association were examined by initiating the reaction with the addition of pre-

warmed (37 °C) or precooled (0.5 °C) PC12 cells to the polystyrene tube containing the prewarmed (37 °C) or precooled (0.5 °C) ^{125}I-βNGF diluted to the appropriate concentration in PBS/BSA. Single samples (100 μL) were taken as rapidly as possible in order to define the early portion of the binding curve accurately. Association times were accurately recorded at the moment the microfuge was started; the approximate time for sedimentation is about 3 s (Sutter et al., 1979), and the time the cells remain in the presence of the free concentration of ^{125}I-βNGF is even shorter. The microfuge tubes were then frozen in a bath of solid carbon dioxide in ethanol, and further processing of the microfuge tubes was carried out after the entire experiment was completed as described (Sutter et al., 1979; Woodruff, 1983). Nonspecific binding was experimentally assayed with the same preparation of PC12 cells and ^{125}I-βNGF. The association time course at 37 °C of this operationally defined nonspecific binding was faster than could be assayed with these techniques, being maximal at the first time point (21 s at 112 pM ^{125}I-βNGF in the presence of a 1010-fold excess of unlabeled βNGF). This level of binding remaining in the presence of a 1000-fold excess of unlabeled βNGF was subtracted from the total binding time course. At 0.5 °C the association rate of the nonspecific binding was considerably slower, with a half-time on the order of 1 min. An independent binding curve in the presence of 1000-fold excess unlabeled βNGF was used to correct each time point to obtain specific binding at the low temperature.

The association data were initially analyzed as an irreversible second-order reaction. The integrated second-order rate equation (Moelwyn-Hughes, 1957) can be rearranged to give a linear function of time with respect to a complex parameter Q, assuming that the contribution from the reverse reaction is negligible:

$$Q = k_1 t = \frac{1}{a-b} \ln \frac{b(a-X)}{a(b-X)} \qquad (2)$$

where a = initial [^{125}I-βNGF], b = initial receptor concentration, and X = [^{125}I-βNGF] bound at time t. The total receptor concentration was experimentally determined from analysis of numerous independent binding curves, each over the full range of radiolabeled βNGF concentrations, by the computerized curve fitting procedure described.

The data were also analyzed as a reversible second-order reaction (Moelwyn-Hughes, 1957) by using parameter W:

$$W = k_1 t = \frac{X_e}{ab - X_e^2} \ln \frac{X_e(XX_e - ab)}{ab(X - X_e)} \qquad (3)$$

where X_e = [^{125}I-βNGF] bound at steady state and a, b, and X are as defined in eq 2. X_e was estimated from the plateau value of bound βNGF at times greater than 60 min in the same experiment.

Dissociation Kinetics. The dissociation kinetics of ^{125}I-βNGF were assayed following a 30-min incubation at 37 °C with PC12 cells. For dissociation at 37 °C, the chase concentration of unlabeled βNGF (greater than a 4000-fold higher concentration) was immediately added. For dissociation at 0.5 °C, however, the cell suspension was placed in ice for an additional 5 min before addition of the unlabeled chase βNGF. Nonspecific binding was estimated from a parallel incubation with at least a 1000-fold excess of unlabeled βNGF present from the initial addition of cells to the ^{125}I-βNGF solution. This parallel assay also received the addition of unlabeled chase βNGF following the 30-min incubation at 37 °C. These nonspecific assays were found to result in no further detectable dissociation following the addition of the chase βNGF, and this low level of binding was subtracted from the total curve. Following the addition of the chase concentration of βNGF at time zero, single samples (100 μL) were removed and processed at recorded times as described for the association data.

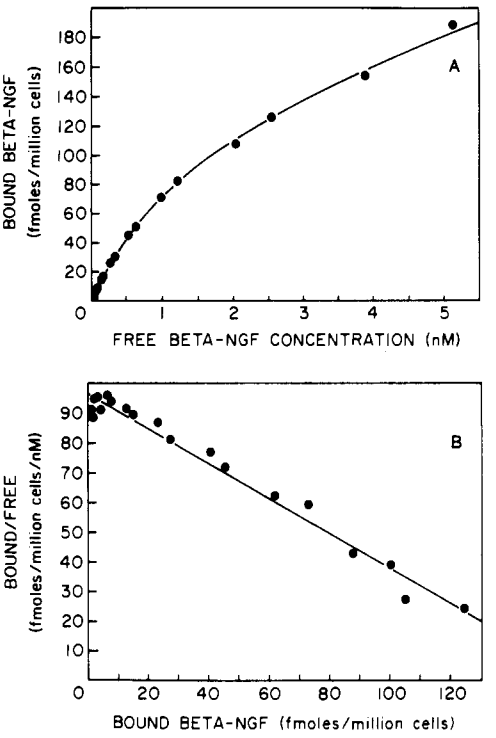

FIGURE 1: βNGF binding at 0.5 °C. The direct binding isotherm (A) and the Scatchard plot (B) are shown for the total specific binding of ^{125}I-βNGF to 1.6 million PC12 cells/mL incubated at 0.5 °C for 100 min. Nonspecific binding was estimated by a parallel incubation with excess unlabeled βNGF and subtracted from the total binding. The solid line was generated from the binding parameters (Table I) of the computer fit of the nontransformed specific binding data with the LIGAND program. Root mean square (RMS) error = 3.8%.

The experimental data were compared to the curve predicted from eq 4 with estimated parameters, and the fit was evaluated by performing a runs test (Mannervik, 1982); parameters were

$$[\beta R]_t = [\beta R_1]_i(e^{-tk_{-1}}) + [\beta R_2]_i(e^{-tk_{-2}}) \qquad (4)$$

varied until the 95% confidence level was attained. In eq 4, $[\beta R]_t$ = concentration of bound ^{125}I-βNGF at time t, $[\beta R_1]_i$ = concentration of site 1 receptor–βNGF complex at initiation of dissociation, $[\beta R_2]_i$ = concentration of site 2 receptor–βNGF complex at initiation of dissociation, k_{-1} = dissociation rate constant for site 1, and k_{-2} = dissociation rate constant for site 2. Data were analyzed and plotted without semilogarithmic transformation in order to preserve the normal variance distribution required for the validity of the statistical testing. The statistics for each fit is given in the appropriate figure legend.

RESULTS

Steady-State βNGF Binding at 0.5 °C. The steady-state binding to PC12 cells was initially investigated at 0.5 °C. The incubation time was set at 100 min since previous work (Schechter & Bothwell, 1981) demonstrated that this was sufficient to establish steady state and this was verified in this study (also see Figure 4). The Scatchard plot (Figure 1) demonstrated the absence of any curvilinearity arising from site heterogeneity in all experiments at this temperature. The K_d for the total binding curve was 1.43 ± 0.12 nM for the

Table I: Summary of βNGF Association with PC12 Cells[a]

	temperature of binding		
	0 °C	37 °C	
Total Specific Receptors			
R_0 (sites/cell)	99 000 ± 7600	155 000[b] ± 18 000	180 000[c] ± 28 000
K_d (nM)	1.7 ± 0.14	0.52 ± 0.03	1.5 ± 0.18
cooperativity parameter			9[d] ± 3.8
nonspecific binding[e] [sites/(cell·pM)]	6.2 ± 0.1	3.3 ± 0.9	
Cold Chase Stable Specific Receptors			
R_0 (sites/cell)			4000[f] ± 800
K_d (pM)			65[f] ± 11
cooperativity parameter (B)			4.0[f,g] ± 2.4
R_0/K_d, nonsaturated [sites/(cell·pM)]			11.1[h] ± 2.1

[a] Parameters and standard errors were obtained from LIGAND analysis of specific binding data shown in Figures 1–3 and 6 except as noted. Sites/cell = 605 × fmol/million cells. [b] Average of four noncooperative experiments. [c] Positively cooperative experiment of Figure 3. [d] The second dissociation constant would be 170 pM. The range of the cooperativity parameter in four experiments was 8–40. [e] Obtained from experimental measurement with excess unlabeled NGF. [f] Parameters obtained from the fit to the binding data for ^{125}I-βNGF remaining after displacement by unlabeled βNGF at 0.5 °C (no nonspecific subtracted) from two combined experiments of the type shown in Figure 6. [g] The second dissociation constant would be 16.3 pM. [h] This ratio of R_0/K_d was obtained from the observed nonsaturated component after experimental correction for nonspecific binding (data not shown) and is consistent with any pair of values of R_0 and K_d to give 11 100 sites/(cell·nM), e.g., 5700 sites/cell and 0.52 nM.

single class of 81 900 ± 8100 sites/cell. The fitted nonspecific binding parameter was 16 ± 2.3 fmol/(million cells·nM). This value agrees closely with the nonspecific binding estimated by coincubation with excess unlabeled βNGF, 10.3 ± 0.15 fmol/(million cells·nM). The final computer fit was characterized by a total root mean square error of 2.5%, indicating that a very good fit was obtained. Models including heterogeneity or positive cooperativity did not provide a statistically better fit to the data by the LIGAND program.

Following correction for the experimentally measured nonspecific binding, the computer fitting procedure found a K_d of 1.7 ± 0.14 nM with the specific binding data. This value is not significantly different from the constant determined for the total binding data, and there was no remaining linear component in the fitted binding equation, indicating that the statistical fitting procedure correctly extracted the nonspecific portion of the βNGF binding. The number of sites per cell, which was also not significantly different from the value determined above, was 99 000 ± 7600 (Table I). Further, analysis of the variance suggested that only a slightly poorer fit to the data (RMS error = 3.8%) was obtained. These values from either analysis are comparable in K_d and intermediate in receptor number when compared with other values reported in the literature from graphical determinations (Herrup & Thoenen, 1979; Chandler et al., 1984).

Steady-State Binding at 37 °C. Experiments examining the steady-state binding of ^{125}I-βNGF to PC12 cells were carried out with a variety of radiolabeled βNGF preparations and many PC12 cell preparations. An incubation time of 40 min was sufficient for attainment of the steady state (Landreth & Shooter, 1980; also see below). The results of seven independent experiments fell into two distinct types of binding curves (Figures 2 and 3). These results, when analyzed and computer fit, were found to be statistically best explained by either a binding class model with positively cooperative interactions (four of the experiments) or a model comprised of

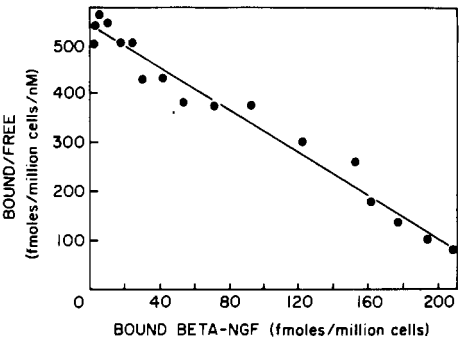

FIGURE 2: Noncooperative βNGF binding at 37 °C. The Scatchard plot is shown for total specific ^{125}I-βNGF binding to 1.43 million PC12 cells/mL at 37 °C for 40 min. Nonspecific binding determined from a parallel assay containing excess unlabeled βNGF was subtracted from the total binding. The solid line was generated from the parameters fit to the nontransformed specific binding data by the LIGAND program. The apparent K_d for this particular experiment was 0.57 ± 0.073 nM, and the number of sites per cell was 157 300 ± 20 500. RMS error = 7.0%.

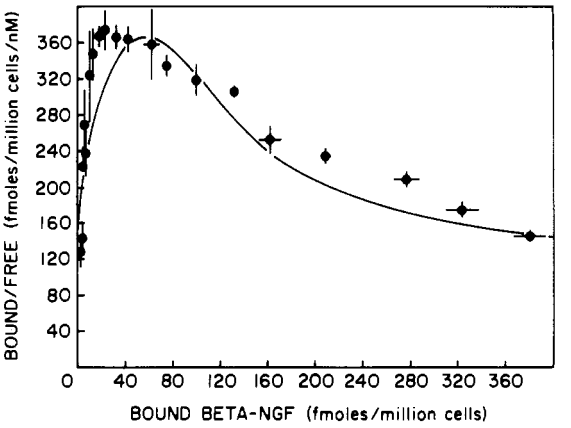

FIGURE 3: Cooperative βNGF binding at 37 °C. The Scatchard plot is shown for total specific ^{125}I-βNGF binding to 1.23 million PC12 cells/mL at 37 °C for 40 min. Nonspecific ^{125}I-βNGF binding was estimated by a parallel incubation with excess unlabeled βNGF and subtracted from the total binding. The vertical and horizontal bars, which are centered on the mean of each triplicate set of samples, represent standard deviations which have been calculated with the appropriate error analysis (Bevington, 1969). The solid line was generated to the computer fit binding parameters (Table I) from the LIGAND program.

only a single, independent class of binding sites (three of the experiments). Figure 2 shows the results obtained from one of the experiments in which the noncooperative model provided the best fit. The four experiments fitting the specific binding data to the noncooperative model, taken together, yield an estimate for the apparent K_d of 0.52 ± 0.03 nM and a binding class size of 155 000 ± 18 000 sites/cell.

Figure 3 shows the typical pronounced downward curvature observed at low bound ^{125}I-βNGF levels in the Scatchard plots of a number of experiments at 37 °C. The curve fitting process produces a significantly better fit with a model that includes positively cooperative interactions. The theoretical line does not exactly match the curvature of the data points in the Scatchard plot (Figure 3) but provides a good visual fit to the untransformed data (not shown). The computer best fit parameters with the specific binding data are 1.5 ± 0.18 nM for the apparent first stoichiometric equilibrium dissociation constant, 9.0 ± 3.8 for the cooperativity parameter, and with a bivalent Adair model (see Materials and Methods) 170 pM for the second dissociation constant. The receptor concentration found for this fit was 180 000 ± 28 000 sites/cell. The range for the calculated apparent second stoichiometric dis-

sociation constant (20–190 pM) from a number of completely independent experiments is close to the βNGF concentration that elicits half-maximal response of neurite outgrowth in the PC12 bioassay (Greene, 1977).

Similar parameters were obtained when either the specific binding data or the total binding data were fit with the LIGAND program in either type of binding curve. Variations in curve shape did not correlate with cell concentration, age of the iodinated βNGF (always less than 2 weeks), or culture conditions (see below). Since the total number of sites found for the two types of curves were not significantly different 155000 vs. 180000, the differences in these sets of observations are reflected solely in the magnitude of the affinity constant and the interactions of the receptor sites. These variable observations may thus reflect a strong dependency of the cooperativity on subtle changes in individual rate constants for binding and/or subsequent steps (see Discussion).

Specific degradation of ^{125}I-βNGF was measured by TCA precipitability in parallel incubations to these binding assays and found to be less than 0.3% of the total NGF added or less than 3% of the bound NGF at 3.3 nM after 40 min. These values correspond to a rate of about 345 pg/(h·10^6 cells) in agreement with Layer and Shooter (1983). Therefore, a small contribution to the steady-state conditions may be attributed to this process. Internalized βNGF (Layer & Shooter, 1983) as measured under comparable conditions by acid stripping (Bernd & Greene, 1984) was less than 10–15% of the NGF bound.[2] These considerations do not appear to account for the variation in type of binding curve observed (see Discussion).

Culture Conditions. Since the range of variation of NGF receptor number on PC12 cells has varied widely in the literature, we investigated several growth conditions and cell lines that might have affected reported results. The effect of different culture conditions was examined with respect to the confluence of the cell culture just prior to harvesting. The two conditions that were selected were a mature culture that was fully confluent and a logarithmic phase culture that was clearly subconfluent. The steady-state binding experiment was done with both cultures simultaneously. The result clearly suggested that the confluence of the culture does not affect the affinity of the receptor. The value for receptor concentration, while not significantly different in the two cases, may not be conclusive since the inaccuracy in cell counting may make the comparison less reliable. The magnitude of the nonspecific binding was found to be slightly greater for the subconfluent cells than for the confluent cells [10.8 and 7.7 fmol/(million cells·nM), respectively]. The model that best fits the data was the same in both cases, namely, one class of sites with positively cooperative interactions.

PC12 cells develop phenotypic variants with a relatively high frequency (Bothwell et al., 1980; Tank & Ham, 1984). In order to evaluate any difference between two PC12 cell sources, a series of four independent experiments was carried out examining the steady-state binding properties of the PC12 cells obtained from two laboratories. The results suggest that no significant difference exists in affinity or the type of steady-state binding between the two cell sources at 37 °C. Differences in receptor number may reflect the greater error in cell counting associated with this parameter. A further test of this conclusion was performed by fitting all the data sets simultaneously but allowing the LIGAND program to adjust the number of sites. The result (Table I) confirms the conclusion derived from the independent fits, supporting the suggestion

[2] T. J. Baribault and K. E. Neet, unpublished observations.

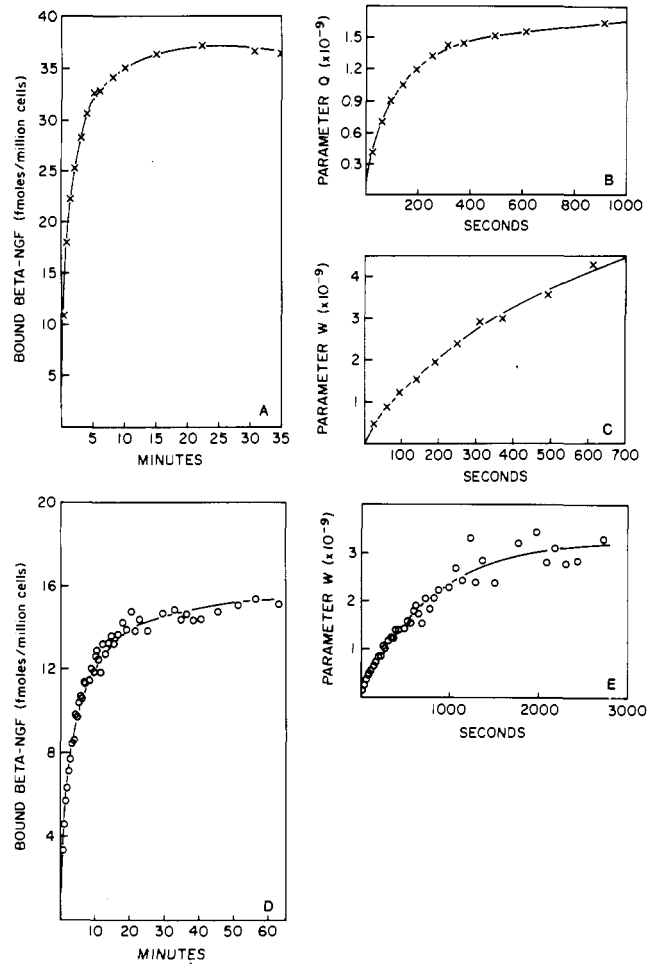

FIGURE 4: Analysis of association kinetics of NGF binding to PC12 cells at 37 °C (A–C) and at 0.5 °C (D and E). (A) ^{125}I-NGF (112 pM) was incubated with 1.58 × 10^6 cells/mL at 37 °C and analyzed for specific binding as a function of time. (B) Replot of the data of panel A for an irreversible, second-order reaction, parameter Q (eq 2). (C) Replot of the data of panel A for a reversible, second-order reaction, parameter W (eq 3). The total receptor concentration was taken as 160000 sites/cell. (D) ^{125}I-NGF (82 pM) was incubated with 1.87 × 10^6 cells/mL at 0.5 °C and analyzed for specific binding as a function of time. (E) Replot of the data of panel D for a reversible, second-order reaction, parameter W (eq 3). The total receptor concentration was taken as 82000 sites/cell.

of common binding properties. No significant difference was observed in cold chase dissociation rate constants (see below) at either 37 or 0 °C for these two cell cultures.

Association Kinetics. The association kinetics of βNGF with its receptors on PC12 cells were examined at 0.5 and 37 °C. Typical association curves (Figure 4) with 112 pM ^{125}I-βNGF at 37 °C or 82 pM at 0.5 °C were initially analyzed by assuming a simple irreversible second-order process. All the experimental quantities required to evaluate the rate expressions are measured during the kinetic experiment except for the total receptor concentration, which must be obtained from extrapolations from steady-state binding experiments. From the experiments of Figures 1–3 the total receptor density was determined to be 258 ± 31 fmol/million PC12 cells (155000 ± 18000 sites/cell) at 37 °C and 160 ± 13 fmol/million cells (99000 ± 600 sites/cell) at 0.5 °C. The total receptor concentration has introduced the greatest error in these types of analyses previously (Cornish-Bowden, 1976). However, the relatively low coefficient of variation, 12%, for receptor density reported here suggests that estimates of association constants from these experiments are reasonable. Moreover, calculations using estimates of receptor concentrations at ±20% of the best

estimate did not appreciably affect the degree of linearity of the resultant plots.

From the distinct nonlinearity of the irreversible second-order relationship parameter Q (eq 2 under Materials and Methods) seen in a typical plot (Figure 4B), it is clear that the irreversible model of the association kinetics is unsuitable, and therefore more complex association processes were considered. Since the curvature appears to be present even at very early times (Figure 4B), the reverse reaction is not expected to be the source of nonlinearity. Nevertheless, the association data were examined by utilizing the reversible second-order integrated rate equation, parameter W (eq 3 under Materials and Methods), to test definitively a simple second-order process. Figure 4C again illustrates a lack of linearity, indicating that a more complex association process must be occurring. Data obtained at 0.5 °C (Figure 4D,E) also indicate a process more complex than reversible, bimolecular binding. This latter result is somewhat surprising since Chandler et al. (1984) and our laboratory[2] have not found a significant amount of a slowly dissociating component upon binding at 0 °C at short times, although Schechter and Bothwell (1981) did observe such a component. The kinetic association analysis may be more sensitive to small amounts of a slow component than equilibrium observation of slow receptor binding. Similar results were obtained at 20 °C (data not shown).

In order to estimate the numerical values for the association constants, assuming parallel binding of two receptors, the apparent association constants were taken from the limiting slopes of the data in Figure 4C,E. The value for the larger association constant will be approached at earlier times, and the value obtained represents the lower bound for the larger association constant. The limiting slope of parameter W at later times in the reaction will give the upper boundary for the estimation of the smaller association constant. These values were 1.4×10^7 M^{-1} s^{-1} for the faster association and 3.8×10^6 M^{-1} s^{-1} for the slower association from an average of four experiments at 37 °C. Corresponding values at 0 °C were 3.5×10^6 M^{-1} s^{-1} and 7.5×10^5 M^{-1} s^{-1}, respectively. Thus, at a minimum, the ratio between these rate constants is 4-fold.

Dissociation Kinetics. The kinetics of dissociation of PC12 cell bound βNGF were examined by utilizing the "chase" and "cold chase" paradigms (Landreth & Shooter, 1980). Figure 5 illustrates the time course of dissociation when the cell suspension is maintained at 37 °C following addition of the unlabeled chase βNGF. The simple case of a first-order dissociation reaction predicts a single exponential decay when the dissociation is initiated by the addition of a chase concentration of unlabeled βNGF. The data obtained cannot be explained by this model, as expected from other studies with PC12 cells and neuronal cells (Sutter et al., 1979; Schechter & Bothwell, 1981; Landreth & Shooter, 1980; Block & Bothwell, 1983; Chandler & Herschman, 1983). One likely model involves the presence of multiple binding components with different dissociation rates. The possibility of a single binding component with negatively cooperative interactions being present is less likely, since the addition of the large excess of chase βNGF would be expected to yield single exponential dissociation kinetics as all receptors are forced to one form [see also Woodruff and Neet (1986)]. Assuming a two-component model, therefore, the analysis of the dissociation curves was performed by subtracting the contribution of the slower component to determine initial estimates for the rate constants. These estimates were used in the integrated rate equation for this model (eq 4) to simulate full dissociation curves (Figure 5). The slower rate constant was about 50-fold lower than

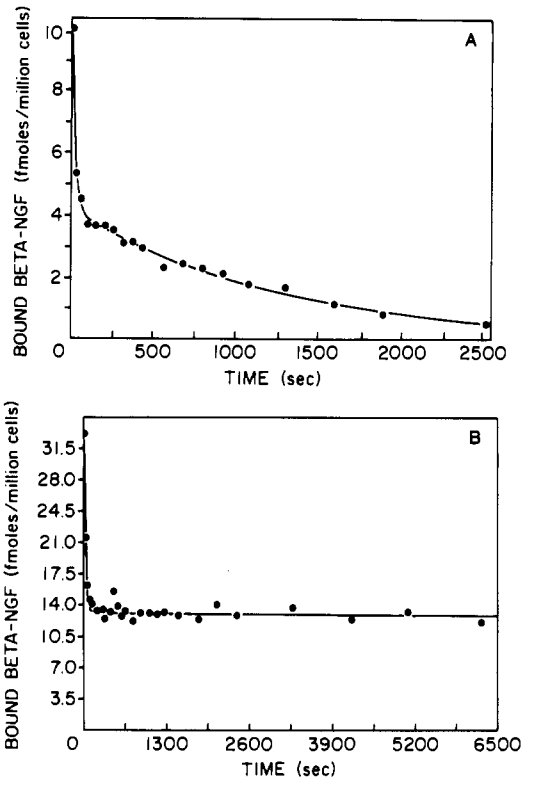

FIGURE 5: Dissociation kinetic data fit to the two-component model. ^{125}I-βNGF was bound to PC12 cells (1.6 million cells/mL) for 35 min at 37 °C and dissociation initiated by 1000-fold excess of unlabeled βNGF. (A) Dissociation at 37 °C. Free βNGF was 30 pM, and the total specific bound ^{125}I-βNGF prior to dissociation was 10.3 fmol/million cells. The fit to eq 4 with the calculated dissociation parameters (Table II) was analyzed by using the runs test and indicated that the residuals are randomly distributed above and below the dissociation predicted from those parameters (solid line). There are ten residuals below and eight above and seven runs. The minimum number of runs (at the 95% confidence level) is six. (B) Dissociation at 0.5 °C. Free βNGF concentration was 76.5 pM, and the total specific binding prior to dissociation was 33.2 fmol/million cells. The fit to eq 4 indicated a random list of residuals (minimum of three runs for the 95% confidence level) with five above and five below and five runs.

Table II: NGF Dissociation Chase Kinetics from PC12 Cells[a]

	temperature of chase dissociation	
	37 °C	0 °C
free [NGF] (pM)	30	76.5
bound [NGF] (pM)	10.3	33.2
$k_{-2} \times 10^3$ (s^{-1})	0.83	0.0032
$k_{-1} \times 10^3$ (s^{-1})	54	43
[BR_1] (pM)	4.2	13.1
% slow receptor	41	40

[a] The data of Figure 5 were analyzed as described under Materials and Methods. The values obtained from the nonparametric fit and used to generate the lines in Figure 5 are listed.

the faster dissociation rate constant (Table II). Examination of PC12 cells from the two sources revealed rate constants that were very similar although there was, at most, a 10% difference in the amount of each component.

The effect of lowering the temperature to 0.5 °C immediately prior to initiation of dissociation was also examined. The time course plotted vs. the logarithm of the fraction remaining bound showed that the dissociation remains complex. Following the same analytical procedure as above, the dissociation rate constants were determined by fitting the data to the two-component model (Figure 5). Table II summarizes

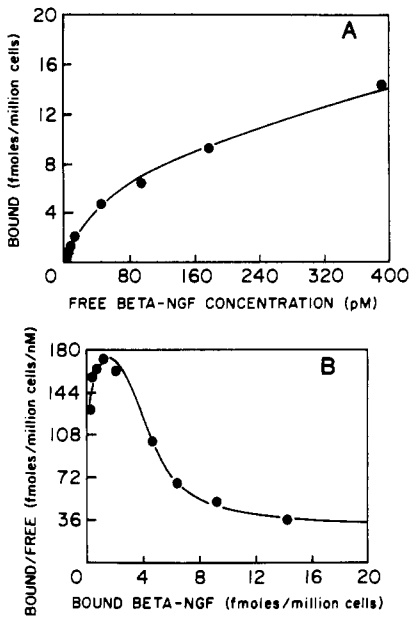

FIGURE 6: Cold chase stable binding. The association of various concentrations of ^{125}I-βNGF progressed for 30 min at 37 °C with a PC12 cell concentration of 1.5 million cells/mL. Cold chase stable binding was that binding that remained 20 min after addition of 0.522 μM unlabeled chase βNGF at 0.5 °C. The binding isotherm (A) and the Scatchard transformation (B) are the original data with no correlation for experimentally defined nonspecific binding. The solid line in each panel represents the parameters obtained from the LIGAND program describing the statistical fit to the data. The fitted parameters for total cold chase stable binding in this particular experiment were as follows: K_d = 48 ± 5; R_0 = 2500 ± 240 sites/cell; cooperativity parameter = 10.3 ± 2.6; nonsaturated binding = 16.4 ± 1.3 sites/(cell·pM). RMS error was 4.5%.

the data obtained. The effect of lowering the chase temperature is to dramatically lower the k_{-2} value while not significantly changing the k_{-1} value, in agreement with the previous observations of Landreth and Shooter (1980) and Schechter and Bothwell (1981).

Cold Chase Stable Binding. By using the greatly increased half-time of dissociation when the temperature is lowered to 0.5 °C, an experiment analogous to a steady-state binding experiment can be performed with the focus on the slowly dissociating component (Schechter & Bothwell, 1981; Landreth & Shooter, 1980; Block & Bothwell, 1983; Chandler & Herschman, 1983). The same methods employed in the kinetic experiments are followed except that only one time point is obtained and that the complete range of ^{125}I-βNGF concentrations used in the steady-state binding experiments are simultaneously assayed. The result of such an experiment is depicted in the direct binding curve and the Scatchard transformation (panels A and B of Figure 6). These complex data were additionally analyzed by computer, utilizing the LIGAND fitting program. The only model that describes the binding curve with reasonable accuracy is one in which there is a significant level of apparent positive cooperativity, as well as additional apparent linear binding component. The pattern was consistently observed in all experiments. When the total binding data, without previous subtraction of the experimentally determined nonspecific binding (Figure 6), are used for the computer analysis, a better fit results than when "specific" binding is experimentally obtained (data not shown). However, the fitted binding parameters (with the exception of the subtracted, nonspecific binding component) are not significantly different between the two analyses. Because of smaller estimated errors, the parameters from the fit to the total appear to be better.

Since this experiment is examining only the cold chase stable sites, the reduction in total binding sites was expected. There is a clear increase in the apparent affinity, regardless of whether the cooperativity parameter is considered. The value obtained for the first apparent K_d was 65 pM when duplicate experiments, including the one in Figure 6, were combined and analyzed by LIGAND, allowing C (eq 1) to normalize the experiments. Schechter and Bothwell (1981) found a similarly complex binding isotherm but assumed a simple single binding class model for their analysis. The linear component, although not well characterized in our experiments that were carried out below 400 pM, after correction for nonspecific binding is consistent with a class of sites with an R_0/K_d of 18 fmol/(million cells·nM) [11.1 sites/(cell·pM)], e.g., with a K_d of 0.52 nM and an R_0 of 9.4 fmol/million cells (5700 sites/cell). The resultant of these two slow classes, about 10000 sites/cell and a 50–500 pM dissociation constant, would not be very different from the single class fit of 15000 sites/cell with the K_d of 200 pM reported by Schechter and Bothwell (1981) or the K_d of 330 pM for the cell surface, high-affinity binding of Bernd and Greene (1984).

The K_d and receptor concentration can also be estimated from a simple assumption of two classes of sites from the association rate constant (Figure 4), the dissociation rate constants (Table II), the total number of receptor sites R_0 (Table I), and the amount of NGF bound to each type of site at a particular concentration. The kinetically predicted values (Woodruff, 1983) are a K_d of 0.19 ± 0.11 nM and an R_0 of 6000 ± 2500 sites/cell for the slow receptor, in reasonable agreement with the values obtained with the cold chase stable steady state (Table I). The K_d estimated for the fast receptor from the kinetic experiments is, however, somewhat higher, 3.2 ± 2.2 nM. Caution must be exercised in applying this analysis, since it is probably an oversimplification (see Discussion). Nevertheless, these calculations lend support to the interpretation that the sites observed with the equilibrium cold chase stable protocol do, in fact, correspond to the kinetically slow-dissociating sites generated at 37 °C.

DISCUSSION

The culture conditions under which cell are grown are known to be very important in a variety of hormonal systems including the NGF–PC12 system. Edgar and Thoenen (1978) have demonstrated that by increasing the confluence of the culture past a certain point the NGF-dependent induction of choline acetyltransferase is lost. We have found, however, that the steady-state equilibrium properties of NGF binding at 37 °C are not altered by varying the confluence conditions of the culture. In agreement with these findings, Herrup and Thoenen (1979) found no significant change in either affinity or NGF receptor concentration in steady-state experiments performed at 0.5 °C with varying confluence. Also, we observed no differences in steady-state binding to PC12 cell lines originating from the same initial isolate (Greene & Tischler, 1976) but obtained by us after adaptation to plastic and maintenance in different laboratories (Schechter & Bothwell, 1981; Landreth & Shooter, 1980). Thus, differences in binding properties reported in the literature (Herrup & Thoenen, 1979; Chandler et al., 1984; Schechter & Bothwell, 1981; Lyons et al., 1983; Landreth & Shooter, 1980; Vale & Shooter, 1985; Bernd & Green, 1984; Edgar & Thoenen, 1978) must be related to more subtle differences in PC12 status or binding methodology. Such alterations in PC12 metabolism might also produce subtle changes in individual rate constants and account for the poor reproducibility of the positive cooperativity (Figure 2 vs. Figure 3).

Steady-state equilibrium results obtained at 0.5 °C yield linear Scatchard plots and are fit adequately under a simple homogeneous binding class model with a K_d of 1.7 nM and 99 000 sites/cell. This K_d agrees well with the values reported by others of 2.9 nM (Herrup & Thoenen, 1979) and 3.2 nM (Chandler et al., 1984), but the number of sites per cell we find is midway between the reported 58 000 and 180 000 (Herrup & Thoenen, 1979; Chandler et al., 1984).

The results obtained at 37 °C are more complex since a pronounced downward curvature was frequently observed in Scatchard plots at the low bound ^{125}I-βNGF region. This kind of a profile could occur artifactually if, at low ^{125}I-βNGF concentrations, sufficient incubation time was not provided to achieve steady-state binding (Clark & Ernest, 1981). This possibility appears unlikely since the incubation times are long when compared to the association time courses, which flatten between 10 and 24 min for a similar range of ^{125}I-βNGF concentrations (Figure 4). If the observed curvature were due to an artifactual lack of steady-state conditions, a shorter incubation time would be expected to enhance the curvature. The result of an experiment with 30-min binding (not shown) was that the curvature was not increased, suggesting that this effect is not artifactually due to excessively short incubation times. Degradation of NGF at this short time was too small to affect this result (Woodruff, 1983; Layer & Shooter, 1983; Chandler & Herschman, 1983; also see above). Internalization of NGF–receptor complex after 30–40 min (Bernd & Greene, 1984) is unlikely to account for the nonlinearity of the 37 °C Scatchard plots since Wiley and Cunningham (1981) have shown that a steady-state model that includes internalization leads to linear Scatchard plots with an altered slope representing a complex steady-state constant. Beck and Goren (1983) have shown by simulations of single-ligand–single-receptor binding at, or near, steady state that the internalization and degradation of ligand–receptor complexes or of ligand alone do not produce Scatchard plots with concave downward, or apparent positively cooperative, curves. We have extended this theoretical approach to a binding model with two independent receptors with the preliminary result that positive optima do not occur in Scatchard plots from independent internalization at one of the two receptors.[3] For extracellular (or cell surface mediated) ligand degradation, longer incubation times will produce concave-upward Scatchard plots. Our experiments, however, show no change in the curvature with longer incubation times, strongly suggesting that these positively cooperative curves were not artifactually produced by internalization and/or degradation phenomena at a single receptor. Furthermore, extensive down-regulation has been reported to require hours in PC12 cells in suspension (Layer & Shooter, 1983; Chandler & Herschman, 1983), although lack of external accessibility can be measured within minutes with PC12 cells attached to collagen-coated plates (Bernd & Greene, 1984). With the PC12 cells grown on plastic plates and studied in suspension in our laboratory, internalization at 100 pM to 1 nM ^{125}I-βNGF in these experiments was less than 10–15% of the bound NGF in 45 min[2] and is interpreted as the linear component of the specific cold chase stable binding (Figure 6, Table I; also see discussion of Figure 7, below). Internalization is much less when measured in suspended cells (Yankner & Shooter, 1979; Schechter & Bothwell, 1981) than it is when measured on plates (Bernd & Greene, 1984) and is highly dependent on culture conditions. Subsequent studies in our laboratory with cells grown differently have found somewhat higher levels of internalized βNGF (20–40%), have studied the concentration dependency, and have determined that acid-stable ligand is less than the cold chase stable binding.[4] A detailed report of these studies will be the subject of a subsequent paper.

The only model that would fit the data having downward curvature in the Scatchard plots was a single class of bivalent binding sites interacting in a positively cooperative fashion. More complex models examined with the LIGAND program, including two classes with or without site–site interactions, were unable to explain the data. The K_d from either type of data reported here, 0.5 or 1.5 nM, is consistent with the range reported at 37 °C with cells, 0.2 nM (Schechter & Bothwell, 1981), or with solubilized membranes, 2.7 nM (Cattaneo et al., 1983) and 5 nM (Lyons et al., 1983). The number of receptors per cell, 160 000–180 000, is higher than the 60 000 found by Schechter and Bothwell (1981) but is similar to that reported by Chandler et al. (1984) at 0 °C. Either the cooperative or noncooperative type of binding data yields the same number of sites in our analysis. Our observation that the number of receptors capable of binding βNGF at 0.5 °C is approximately half of the number available at 37 °C is too large a difference to be accounted for simply by increased internalization at the higher temperature and suggests the interesting possibility that a subunit of a dimeric receptor, or a receptor capable of dimerizing, is cryptic at the reduced temperature. These NGF data and interpretation are also reminiscent of recent binding data of insulin to hepatoma cells (Marsh et al., 1984) that provide evidence for a positively cooperative component with the insulin receptor. Our ability to discern this cooperative component may be due to the emphasis on the low concentration range of NGF used in these experiments.

Dissociation rate experiments presented here agree very well with both the results by Landreth and Shooter (1980) and those by Schechter and Bothwell (1981). All groups observed the same temperature effect on the slowly dissociating class of sites. The 4-fold difference in rates of association reported by Schechter and Bothwell is not based on measured association rate constants but rather on the relationship of the observed steady-state binding constants. If the observed association data is the result of two independent rate constants, then the limits can be estimated from the extremes of the binding association time course as explained earlier (Figure 4). The results presented here suggest that the apparent association rate constants are not identical, and we have estimated that at least a 4-fold difference exists between them at either 0, 20, or 37 °C. A number of investigators have assumed that rate constants in this range indicated a diffusion-limited reaction (Sutter et al., 1979); however, the rate constant expected for a diffusion-limited association between a protein and cell surface receptors is not known precisely. The clear implication of the present experiments is that at least the slower association is limited by another process, slower than diffusion. Since this relationship was also observed at low temperature (Figure 4) where active cellular processes are not occurring, it must reflect membrane phenomena. The existence of association processes slower than the diffusion limit during the binding of βNGF to PC12 cells is supported by a parallel finding with the association of βNGF to dorsal root ganglionic cells (Hawrot, 1982; Stach & Wagner, 1982).

Because the plot for the second-order, reversible reaction is nonlinear, a multistep binding must be occurring, regardless

[3] N. R. Woodruff and K. E. Neet, unpublished results.

[4] M. Kasaian and K. E. Neet, unpublished results.

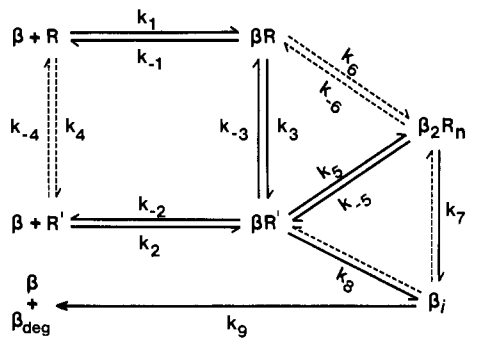

FIGURE 7: General binding model. β denotes βNGF, R and R′ denote two states or forms of the receptor, and β_2R_n denotes two molecules of β binding to a bivalent (or higher) receptor. β_i and β_{deg} are internalized and degraded forms of βNGF, respectively. Equilibria 3 and 4 are isomerizations, and equilibria 1, 2, 5, and 6 are ligand associations. Dashed lines represent reactions that are less certain.

of whether the first encounter complex is only diffusion limited. This complex binding could be either concurrent binding to two distinct receptors or receptor sites; a sequential step with the initial NGF–receptor complex undergoing a subsequent isomerization, aggregation, or alteration; or both the presence of two receptors and isomerization (Figure 7). Our data indicate that a complex association must be occurring independently of energy metabolism, at least partially, because the association kinetics appear more complex than a reversible, bimolecular complex formation at 0 °C. For these reasons and because of the positive cooperativity, simple comparison of equilibrium constants with the ratio of association to dissociation rate constants is probably inappropriate at this time. Preliminary assessment of the shape of Scatchard plots from measured rate constants has suggested that the observed monophasic curves for 37 °C binding at steady state may be within the expected experimental error of the multicomponent system (Woodruff, 1983). A more quantitative analysis of rate constants is in progress and will be presented in a subsequent publication.[3]

A general model consistent with all of the data presented here is shown in Figure 7. The square, equilibria 1–4, represents the initial binding of βNGF to each of two receptor forms, R and R′. βR and equilibrium 1 would represent the "fast" receptor. The conversion between the two receptor forms is shown predominantly as step 3, enhanced by the binding of βNGF, i.e., $k_3/k_{-3} > k_4/k_{-4}$. Step 4 is shown as dashed lines because there is no existing evidence that suggests interconversion of the receptors occurs without ligand or other agent. Our data do not offer a choice between the preexisting two-receptor model, K_1 and K_2 with K_3 and K_4 negligible (Schechter & Bothwell, 1981), and the conversion model, K_1 followed by K_3 with K_2 and K_4 negligible (Landreth & Shooter, 1980). Either of these two-step models (K_1 plus K_3, or K_1 plus K_2) will satisfactorily explain[3] the nonlinearity of the parameter W kinetic association data of Figure 3 at 0 °C. β_2R_n is included as the simplest way to explain the positive cooperativity observed, and the two steps, K_1 and K_6, are equivalent to the bivalent Adair model discussed for the fitting (eq 1A and 1B); whether the receptor is initially bivalent or dimerizes after βNGF binding is indeterminant. The binding of step 6 is shown as a dashed line because of the variability of the observation of cooperativity in binding in the total ligand data; presumably the relationships between K_1, K_6, and K_3 are the steps sensitive to culture or binding conditions at 37 °C and cause the observation of cooperativity in only about half of the experiments with total specific binding (Figures 2 and 3). These reactions are being modeled to determine the inherent sensitivity.[3] Step 5, however, represents the positive cooperativity consistently seen in the slowly dissociating ligand (Figure 6). Dissociation through k_{-2}, k_{-3}, k_{-5}, or k_{-6} would each be sufficiently slow to include all of these forms in the slowly dissociating, externally bound βNGF. Cold chase stable ligand would include βR′, β_2R_n, and β_i. β_i represents the truly "internalized" ligand, with or without bound receptor, which can be released to the extracellular medium (K_9) either intact, β, or degraded, β_{deg}, to establish the criteria for the steady state (Figure 4) of the analysis. Not shown in Figure 7 is the recycling of the receptor, de novo insertion of receptor into the plasma membrane, and receptor degradation that may also occur; these receptor processes are included in steady-state systems (Wiley & Cunningham, 1981) that are being used for modeling of the NGF–receptor system.[3] Internalization is shown only from βR′ (k_8) or β_2R_n (k_7) because of the data of Bernd and Green (1984) indicating that the high-affinity binding is involved; whether this endocytosis comes from step 7 or 8 or both cannot yet be determined. Total cell-associated βNGF that we have measured in these experiments would include βR, βR′, β_2R_n, and β_i. Signaling could come from any one or more of these species, although the most likely would appear to be βR′ or β_2R_n, on the basis of the concentration dependency. At 0 °C only βR and βR′ exist; i.e., binding is complex but noncooperative and not internalized.

The best values that we can assign from our steady-state analysis at 37 °C are as follows. The total cell-associated βNGF (βR + βR′ + β_2R_n + β_i) is 155000 molecules/cell with an apparent average steady-state K_d of 0.52 nM. β_2R_n is 4000 molecules/cell with a first K_d of 65 pM. The maximum of the remaining slowly dissociating ligand (primarily β_i) would be 5700 molecules/cell with a corresponding K_d value of 0.5 nM (Table I). Estimated dissociation rate constants would be $k_{-1} = 5.4 \times 10^{-2}$ s^{-1} and k_{-2} or k_{-3} (since k_{-1} is fast) = 8.4×10^{-4} s^{-1}. Limiting association rate constants would be $k_1 = 1.4 \times 10^7$ M^{-1} s^{-1} and $k_2 = 3.8 \times 10^6$ M^{-1} s^{-1}. Estimates of degradation under our conditions (Woodruff, 1983) suggest that k_9 must be greater than 8×10^{-5} s^{-1}, a hydrolytic rate constant somewhat less than that observed for epidermal growth factor (EGF) with fibroblasts (Wiley & Cunningham, 1981).

Because of the complexity of the system and the simplification of the present analysis, the ratio of these "off" and "on" rate constants for the two "sites", 3.2 nM and 0.2 nM, respectively, do not agree adequately with constants measured from binding (Table I), confirming that the binding studies yield steady-state constants and not simple equilibrium dissociation constants (Wiley & Cunningham, 1981). Further analyses will be required.[3] Nevertheless, this working model semiquantitatively explains all of our data and is consistent with the other pertinent data in the literature. The model is a minimal one except for the inclusion of R′ as an unoccupied receptor form and the inclusion of both step 7 and step 8, only one of which is required to explain internalization. Other, similarly complex models may also explain most of the observations, but this model provides a reasonable basis on which to design further experiments and with which to obtain quantitative estimates of constants.

The positive cooperativity observed in our cold chase experiments has been fit to the divalent receptor model (Feldman et al., 1972; Munson, 1983) and requires a mechanism such as that indicated by the β_2R_n complex (Figure 7). Specific models that also generate positively cooperative interactions include NGF as a dimeric ligand, dimerization of the NGF–receptor complex itself, and clustering of the complex in the

membrane. The clustering of βNGF–receptor complexes has some support from the studies that utilized photobleaching of fluorescent labels (Levi et al., 1980) and photoaffinity cross-linking (Hosang & Shooter, 1985; Buxser et al., 1985). Further, the complexity is more easily explained if the cooperative interactions are the result of dimerization (Buxser et al., 1985), association with other membrane components (Hosang & Shooter, 1985), or association with cytoskeletal components (Schechter & Bothwell, 1981; Vale & Shooter, 1982). A model involving clustering or other membrane events would also explain the temperature effect observed. The elimination of positively cooperative interactions when the binding is performed at 0.5 °C correlates with the known changes in membrane fluidity at low temperature (Schlessinger et al., 1978; Schechter et al., 1979). The results presented here are consistent with a physiological role for an early event of the NGF–receptor complex in the membrane such as microaggregation. The apparent positively cooperative interactions, which occur in the same range of βNGF concentrations as do the neurite-promoting effects in PC12 cells, are eliminated in the cold. The temperature dependence of the dissociation rate constant for the slower PC12 component may be more sensitive to the membrane changes with decreasing temperature than is the faster component as a result of membrane microaggregation involvement.

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