We analyzed the kinetics and thermodynamics of $^{125}$I-NGF binding to NGF-receptor on PC12 cells. We used conditions of pseudo-first order kinetics and techniques to quantitate internalized complexes, "slow" or high affinity binding complexes, and cell surface "fast" or low affinity complexes. Two possible models were examined: binding to two independent receptors at the cell surface (i.e. high and low affinity forms of NGF-receptor) and a model for consecutive formation of fast, low affinity binding followed by slow, high affinity binding or internalization. Our data are consistent with the consecutive model only. The rates of association and dissociation of NGF with slow, high affinity sites and internalized, acid wash-resistant sites are indistinguishable from each other. We also analyzed, in detail, the two assays primarily used to distinguish slow binding complexes from internalized complexes. Scatchard analysis of total binding and dissociation of pre-equilibrated $^{125}$I-NGF in the presence of unlabeled NGF at high concentration (cold wash). Neither of these assays shows any evidence that the slow, high affinity binding step is different from internalization of the $^{125}$I-NGF-receptor complex. Based on this analysis, there are only two detectable forms of NGF-receptor on PC12 cells: complexes on the surface of the cells with a binding affinity of 0.5 nM at 37 °C and complexes internalized by the cells. Furthermore, the data are consistent with a model in which NGF-receptor is internalized constitutively and independently of occupancy by NGF. We also examined the fate of internalized $^{125}$I-NGF. In the first 60 min after contact with PC12 cells, no degradation of $^{125}$I-NGF was observed. Moreover, a significant amount of $^{125}$I-NGF recirculates to the cell surface and is released as intact, $M_r = 13,000$ NGF. The cells were also stimulated by NGF in a primary neurite outgrowth assay with an ED$_{50}$ of 2–16 pm under conditions of low initial cell numbers in a large extracellular volume of NGF-containing medium. Thus, low level occupancy of the cell surface receptors, $K_d = 0.5$ nM, for several days is sufficient to stimulate neurite outgrowth. This indicates the presence of sparse NGF-receptors on the surface PC12 cells.

The binding of NGF to its specific receptor on PC12 cells is inconsistent with a simple, single step interaction of NGF with receptor (1–10). The most striking demonstration of the complexity of binding kinetics is observed when $^{125}$I-NGF, bound to PC12 cells after incubation at 37 °C, is dissociated from those cells; the dissociation is clearly biphasic (1, 2, 4, 6). Observation of deviation from linear Scatchard plots has also been used to support the argument that more than one form of NGF-receptor is present (1, 3–5, 8–10). Detailed kinetics of aggregate NGF complex formation and dissociation from NGF-receptor in PC12 cells were recently presented by Woodruff and Neet (1). Both association and dissociation kinetics showed the presence of at least two distinct complexes. It appears that the weaker complex forms and decomposes rapidly, whereas the high affinity complex forms and decomposes slowly. In the literature, as well as in the following discussion, it is important to note that several terms are used to describe each of the two forms of NGF-receptors. Low affinity, "fast" and type II receptor refer to one form, and high affinity, "slow" and type I receptor refer to another form of receptor.

A major unresolved issue is the relationship between high and low affinity binding. A priori, the presence of two distinct sites can be rationalized either by the presence of two distinct and independent forms of receptor on the surface of the cells or by a pathway where a free receptor on the surface of the cell forms first a low affinity complex which then transforms to the high affinity form (1). To date, the experimental evidence for neither model is compelling. The two pathways should, in principle, be distinguishable both by thermodynamic and kinetic methods. In fact, parallel pathways should yield two distinct equilibrium binding curves, whereas the consecutive pathway should yield only one binding curve once the slower equilibrium is established. However, in practice, the notorious sensitivity of Scatchard plots to slight experimental errors and the experimental difficulties inherent in measuring slow binding in cultured cells made the results to date ambiguous. The kinetic methods, on the other hand, would only distinguish between the two pathways if the kinetics of formation and decomposition of the two complexes could be measured independently instead of measuring only the sum of the two concurrently.

Interpretation of data is further complicated by partial internalization of the NGF-receptor complex at physiological temperatures. That internalized complexes do occur is not in dispute. A scheme describing internalization of $^{125}$I-NGF mediated only by high affinity receptors has been proposed (3). In order to distinguish between high affinity and internalized receptors, they must be quantitated separately and unambiguously. Equilibration with $^{125}$I-NGF at 37 °C followed by incubation at 0 °C with a high concentration of unlabeled NGF does not necessarily distinguish high affinity binding from internalization, since a high external concentration of unlabeled NGF cannot compete for binding at internalized
125I-NGF-NGF complexes. This approach is not sufficient to distinguish the forms. Similarly, Scatchard plots are affected by internalization as well as by the presence of more than one form of receptor and do not allow quantitative distinctions to be drawn. Even observing the difference between 125I-NGF retained by cells after acid washing compared to the amount released following incubation with a high concentration of unlabeled NGF is not sufficient, if nonspecific binding under each washing condition is not rigorously accounted for.

At the onset of this work, we felt that measuring the binding equilibria and the kinetics of formation and decomposition of the two complexes individually and separately should establish whether NGF binds by the independent or the consecutive mechanism. The rapid decomposition of the cell surface NGF-receptor complexes in acidic media or after dilution in the presence of a high concentration of unlabeled NGF at 0 °C provided two tools to assess the concentration of internalized and slow or high affinity binding, respectively. However, as pointed out above, internalized complexes are not always distinguishable from high affinity binding as assessed using these techniques. This leaves open the second major question regarding the binding kinetics of NGF. Is there a relationship between high affinity binding and internalized complexes? Nevertheless, these approaches do allow a comparison to be drawn between the rate of accumulation of internal complexes and slow, high affinity binding.

Regardless of which dissociation conditions are chosen, the extrapolation of the binding kinetics to infinite time should yield reliable values for the concentration of the high and low affinity complexes. With these tools, it was possible to analyze the kinetics and thermodynamics of the formation of complexes of NGF with receptor.

PC12 cells represent an accepted model, not only for the study of binding of NGF to its specific receptor (1–3, 5, 7), but are also used as a model for the biology of neural differentiation. PC12 cells also provide a rich source of luminal cells without the limitations of material inherent in systems dependent on harvesting cells as primary cultures of sympathetic neurons from animals.

In the following report, we propose to show that the kinetics and thermodynamics of binding of 125I-NGF to PC12 cells are only consistent with the initial formation of a single type of cell surface NGF-receptor complex known variously as fast, low affinity or type II binding. Consecutively, a portion of this fast complex is then moved to an internal compartment. Furthermore, we present data which indicate that the slow binding complexes are not distinct from internalized NGF-receptor complexes. Measurement of slow complexes is complicated by nonspecific binding such that there is no compelling evidence to support the conclusion that slow binding is anything other than internalization of the NGF-receptor complex. In this regard, our work is consistent with the work of Eveleth and Bradshaw (34). They observed that type I (slow or high affinity) and type II (fast or low affinity) NGF-receptor species can be interconverted by changing the position of the receptor from the surface to an internal compartment. In our work, we used a different experimental approach and report the kinetics of the initial cell surface binding and relate that to the conversion to internalized (type I, high affinity, or slow) binding complexes. The results from both of these experimental approaches support the same general conclusions regarding the properties of NGF-receptor. Thus, the heterogeneity of NGF binding arises not from the presence of two different species of receptor in the cells, but is a consequence of the pathway by which NGF-receptor complex is processed in the cell.

**RESULTS**

To begin examination of the interaction between NGF and its receptor(s) on PC12 cells, we observed the binding of NGF over a 10-fold range of NGF concentrations. For these experiments we used a single, low concentration of 125I-NGF for each experiment (0.01 nM, 0.092 nM, or 0.1 nM) and 13–19 concentrations of unlabeled NGF ranging from 0.004 nM to 1200 nM. Results from 0.01 nM and 0.1 nM 125I-NGF are shown in Fig. 1. The use of this broad range of concentrations of unlabeled NGF allows detection of binding sites with affinities across the relevant range of NGF concentrations. Furthermore, the results of this approach can be plotted and examined directly without resorting to Scatchard plots. The existence of two independent sites should result in a double sigmoidal plot. The highest affinity sites were evaluated using a weighted nonlinear analysis of the Langmuir binding isotherm determined from data pooled from all three concentrations of 125I-NGF that are given in Table 1A. Only one species of sites with a single affinity was detectable in the relevant range of NGF concentrations. Fits of the experimental data to a model of two binding sites did not improve the congruency of the experimental points with the theoretical curve. These results alone demonstrate that the presence of more than one form of NGF-receptor cannot be due to independent species of binding sites.

We also observed the effect of temperature on the binding of 125I-NGF. As seen in Table 1A, the affinity of 125I-NGF for PC12 cells is lower at 0 °C than at 37 °C. This observation agrees with those of Woodruff and Neet (1). It is consistent with a model where the interaction of NGF with its specific receptor is mediated predominantly by hydrophobic interactions.

We followed up the equilibrium binding studies by observ-
The kinetics of association of $^{125}$I-NGF with intact PC12 cells. We used three different concentrations of $^{125}$I-NGF under conditions sufficient to satisfy requirements for pseudofirst order kinetics, i.e. less than 10% of the $^{125}$I-NGF was consumed in the binding reaction. The pattern for total binding (open circles) of $^{125}$I-NGF shown in Figs. 2 and 7 was consistently observed. The early phase of the data for total binding (circles) was analyzed using an integrated equation for first order kinetics. The results are consistent with pseudofirst order kinetics as shown by the agreement of the experimental points and the calculated theoretical curves (Figs. 2 and 7). We observed a linear dependency of $k_{\text{exp}}$ on the concentration of $^{125}$I-NGF. If the early phase of the binding is a one-step equilibrium, then the experimental rate constant ($k_{\text{exp}}$) of the forward reaction should be given by $k_{\text{exp}} = k_1 + [\text{NGF}]$ $k_1$, these results translate to a $k_1 = 0.025 \pm 0.004 \, \text{nM}^{-1} \, \text{s}^{-1}$ and a $k_2 = 0.0079 \pm 0.0009 \, \text{s}^{-1}$. These were used to calculate an equilibrium dissociation constant of $K_d = 0.33 \pm 0.1 \, \text{nM}$. We also measured, over a limited range of radioactive ligand, the equilibrium binding of $^{125}$I-NGF to the receptor. The data were again analyzed according to the scheme of a one-step equilibrium, $i.e.$

$$^{125}\text{I-NGF bound} = \frac{D_{diss} \cdot [^{125}\text{I-NGF}]}{K_d + [^{125}\text{I-NGF}]}$$

We found that the data were consistent with this equation, and we calculated $K_d = 0.60 \pm 0.14 \, \text{nM}$. This value is consistent with the $K_d$ derived from the kinetic parameters (Table 1B) and with the $K_d$ derived from isotope dilution experiments (Fig. 1). In conclusion, both the kinetic and equilibrium binding experiments characterizing the fast binding are consistent with a single, diffusion-limited reaction between $^{125}$I-NGF and a single form of receptor on the surface of PC12 cells.

Although our data are consistent with rapid complex formation involving a single species of receptor during the first 60 s after mixing $^{125}$I-NGF with NGF-receptor on PC12 cells, incubation over a 30-45 min interval results in a gradual additional accumulation of cell-associated $^{125}$I-NGF. This may be explained by association with a second species of receptor over this prolonged period of time or by conversion of some of the rapidly associated receptor to a different state. This could include internalization of the $^{125}$I-NGF-NGF-receptor complex.

First, we observed the amount and proportion of $^{125}$I-NGF internalized after incubation at $37 \, ^\circ C$ for 1 h. As the concentration of NGF increased, the amount of NGF internalized also increased (Fig. 3A). However, the proportion of bound NGF internalized was constant. Although a slight negative slope is evident in Fig. 3B, statistical analysis indicates that the values are not significantly different over the 300-fold range of concentrations tested. Thus, 31 ± 11% of receptor was internalized during a 1-h incubation, regardless of the concentration of NGF used. This result indicates that internalization of NGF-receptor is independent of occupancy by NGF. Additionally, these data provide support for Model II. If Model I were true and internalization was mediated by only one of two independent forms of NGF-receptor with different affinities, internalization would be dependent on occupancy

| Table I | Kinetic and equilibrium binding parameters for $^{125}$I-NGF binding to PC12 cells |
|---------|---------------------------------|
| A. Equilibrium dissociation constants. | |
| Experiment no. | Figure | Incubation temperature | $K_d$ | Sites/cell |
| 1a | 1 | 37  | 0.46 ± 0.11 | 27,000 ± 5,600 |
| 1b | 1 | 0 | 1.1 ± 0.45 | 18,000 ± 7,500 |
| 2a | 9 | 22 | 0.72 ± 0.078 | 49,000 ± 2,600 |
| 2b | 9 | 22 | 0.65 ± 0.059 | 63,000 ± 2,800 |
| 2c | 9 | 22 | 0.49 ± 0.091 | 63,000 ± 6,000 |
| B. Experimental rate constants for binding of $^{125}$I-NGF to PC12 cells* | |
| Concentration of $^{125}$I-NGF | Results from n experiments | $k_{exp}$ |
| nM | fmol | s$^{-1}$ | 10$^5$ |
| 0.1 | 6 | 10.2 ± 0.8 |
| 0.2 | 3 | 13.4 ± 2.1 |
| 0.3 | 2 | 16.2 ± 2.3 |
| C. Equilibrium dissociation constant calculated from values determined from the maximum (infinite time) binding** | |
| Concentration of $^{125}$I-NGF | $K_d$ |
| nM | fmol |
| 0.05 | 0.64 |
| 0.1 | 1.03 |
| 0.2 | 1.83 |
| 0.3 | 2.49 |

* Fitted by linear regression to the equation $k_{exp} = k_1 + [\text{NGF}] \cdot k_1$, these results translate to a $k_1 = 0.025 \pm 0.004 \, \text{nM}^{-1} \, \text{s}^{-1}$ and a $k_2 = 0.0079 \pm 0.0009 \, \text{s}^{-1}$. These were used to calculate an equilibrium dissociation constant of $K_d = 0.33 \pm 0.1 \, \text{nM}$.

** $K_d = 0.60 \pm 0.14 \, \text{nM}$ (see text).
The internal compartment appears more complex than the receptor should be maximal, whereas according to Model II, there is little or no binding. According to Model I, the initial rate of internalization is rapid and efficient. As shown in Fig. 4, the association of ligand.

Statistical analysis (analysis of variance) showed that during the first 40-60 s after addition of \(^{125}\)I-NGF to the cells, the amount of internalized ligand rapidly appeared extracellularly. The amount released following a wash with acetic acid and salt did not differ significantly from the amount spontaneously released upon filtration. Incubation of the cells was then continued for 60 min at either 37 °C (or 0 °C as control) to allow processing and/or intracellular circulation. As shown in Fig. 5, a significant proportion of the previously internalized \(^{125}\)I-NGF rapidly appeared extracellularly. The amount spontaneously released did not differ significantly from the amount released following a wash with acetic acid and salt. As determined by SDS-PAGE (Fig. 5B), the \(^{125}\)I-NGF released during this time course was intact NGF with \(M_r = 13,000\). From these results it appears that a significant proportion of the \(^{125}\)I-NGF can bypass a lysosomal degradation pathway in the cell and can recirculate to the cell surface after internalization where it can be released into the extracellular space.

We conclude from the studies described above that Model II is the appropriate model to describe binding to NGF-receptor. We also conclude that the second step is considerably slower than the first, making it possible to observe the second step largely independent of the first step. We observed that the second step of the reaction does not go to completion: i.e. approximately 30% of the receptor is internalized. This indicates that NGF-receptor is not only going
The third set of triplicate samples was removed from the 37°C incubation, aliquots: the incubation for 1 aliquot was continued at 0°C; the 2nd ice cold KRH/A over a 10-min period. The solution was split into 2 aliquot was transferred to a 37°C water bath. Incubation at these temperatures was continued for a total of 60 additional min. 100-p1 buffer was added to the supernatants to produce final concentrations of 0.1 mM choline Cl and 50 mM dithiothreitol. A third set of triplicate samples was removed from the 37°C incubation, brought to 150 mM choline Cl and 50 mM acetic acid (pH 2.5), and incubated on ice for 10 min before centrifugation and processing as described immediately above. The values shown in A are the mean cpm of 125I-NGF in the supernatants after incubation at 0°C (circles), 37°C (triangles), or incubation at 37°C followed by the acid wash procedure (squares). B, 50-μl samples from the 37°C incubation (after washing extracellular 125I-NGF with KRH/A and corresponding to the triangles in part A, above) were run on a 15% polyacrylamide gel. The time after initiation of the incubation period at 37°C is given above the appropriate lane on the gel. The dye front is at the bottom of the photograph.

We used the constants for association and dissociation from cell surface receptors and internalization and externalization of receptors derived from the kinetic experiments in these equations. Since the integrated form of this system of differential equations is too complex for use in this analysis, fifth order Runge-Kutta numerical integration was used to determine integrated values for these equations, and the resulting curves were superimposed on data generated in an experimental data set of binding and internalization. As shown in Fig. 6, the results from the numerical integration of these differential equations are in substantial agreement with the experimental data. This provides experimental support for the model.

A distinction had been made between the high affinity (slow) NGF-receptor and internalized receptor (2, 3, 5, 10, 13). We set out to test whether we could distinguish between the early time course of internalization and the early time course of binding to the slow binding component. As shown in Fig. 7, the binding of 125I-NGF to slow NGF-receptor on PC12 cells is very similar to the accumulation of internalized 125I-NGF-NGF-receptor complex. In five independent exper-
To equilibrate both the internal and external compartments, NGF with PC12 cells was incubated in 100 μl of KRH/A containing 0.1 nm 125I-NGF for 30-45 min at 37 °C. Nonspecific binding control samples also contained 75 nM unlabeled NGF. Individual samples were diluted with 4 ml of ice cold KRH/A containing 75 nM unlabeled NGF, but additionally were brought to a final concentration of 0.15 M choline Cl + 50 mM acetate (pH 2.5), and the incubation on ice was continued for an additional 2 min before filtration. The values shown are the means of triplicate samples from a single experiment. The curves shown are theoretical curves generated from the parameters for the sum of two exponentials calculated from the data shown using the equation given in the text. A duplicate experiment produced nearly identical results, and those results were combined with the results from the experiment shown to generate the values presented in the text.

A statistically significant (p < 0.05) 40-60-s delay was observed after addition of 125I-NGF before significant slow receptor binding was detected. Thus, both internal and slow binding were detected after a statistically significant 40-60-s delay following addition of 125I-NGF to the cells. Additionally, the k values calculated from internal and slow binding experiments were not significantly different from each other. Thus, there is no obvious kinetic difference between the association to the slow binding sites and the rate of internalization of NGF-NGF-receptor complex.

We also tried to distinguish between the rate of dissociation of slow binding and the rate at which internalized receptor reappears on the surface of PC12 cells and the 125I-NGF-NGF receptor complex subsequently dissociates. This was done by binding 125I-NGF to equilibrium at 37 °C for 30 min to equilibrate both the internal and external compartments. Next, the cells were diluted 20-fold into KRH/A containing 70 nM unlabeled NGF and incubated for time intervals, as indicated, at 37 °C. One set of samples was rapidly filtered at each of the times indicated. This treatment should result in dissociation from all of the fast binding sites and should allow measurement of the dissociation rate from the slow sites at 37 °C. The second set of samples was also diluted into KRH/A containing 70 nM NGF, but additionally was brought to a final concentration of 0.15 M choline Cl + 50 mM acetate (pH 3.5) for 2 min before filtration in order to remove any slow binding which was not also internalized. In other words, this procedure measured the rate of return of 125I-NGF to the cell surface. As shown in Fig. 8, the dissociation from slow receptor is not significantly different from the return of internalized receptor to the cell surface. The data from both experiments were fit to the following equation:

\[ \text{cpm bound} = C_1 \cdot \exp (-t \cdot k_1) + C_2 \cdot \exp (-t \cdot k_2) \]

where \( C_1 \) and \( C_2 \) represent the capacities of each site, \( k_1 \) and \( k_2 \) represent dissociation rate constants, and \( t \) is time after dilution. The rapid dissociation phase indicates dissociation rates of 0.014 (±0.003) s⁻¹ and 0.020 (±0.003) s⁻¹ for the slow and internal experiments, respectively. These values are not significantly different from the values calculated for \( k_1 \) presented above. Assuming a two-step dissociation, the rate constants for the second phase of the dissociation are 0.023 (±0.002) min⁻¹ and 0.021 (±0.002) min⁻¹ for slow and internal dissociations, respectively. Within a factor of 2, these values agree with values presented previously by Woodruff and Neet (1) for the slow phase of dissociation. More importantly, these values do not significantly differ from each other, in support of the idea that dissociation from slow and internal receptor do not differ.

The values for the slower phase dissociation rate constants calculated from the data shown in Fig. 8, 0.02 min⁻¹, do differ significantly from those calculated from the initial rates of internalization, 0.18 min⁻¹, as described for data shown in Fig. 2. Therefore, the dissociation data were also fit to an equation of the sum of three exponential terms imposing the rate constants for \( k_1 \) and \( k_0 \) derived from the kinetic data. The dissociation data are fully consistent with this model containing a third component which is substantially slower than those corresponding to \( k_1 \) and \( k_0 \). The origin of this third dissociation component is not understood. However, we did note that during the course of the incubation before beginning dissociation a significant degree of cell clumping occurs. Partitioning of some 125I-NGF into the space between cells in clumps could explain the appearance of this third dissociation component. Further experiments will be required in order to determine precisely the origin of this component and, for the present, we cannot totally eliminate the possibility that it has some biological relevance. However, the presence of this third component with a very slow dissociation
rate and observed only in this experimental protocol does not alter the major conclusion from these experiments. Dissocia-
tion from the slow component of binding defined by resistance
to dissociation in the presence of a high concentration of
unlabeled NGF is indistinguishable from dissociation from
internalized sites defined by resistance to dissociation in the
presence of an acidic wash.

DISCUSSION

Analysis of either the kinetics or the thermodynamics of
fast binding indicates the presence of a single type of binding
site with an equilibrium dissociation binding constant of
approximately 0.5 nM. The initial binding of 125I-NGF to
receptor on the PC12 cell surface is completely explained by
a simple, first order mechanism. A second type of site was
detected beginning approximately 60 s after contact with 125I-
NGF. This second type of site can be explained by internali-
tion from the slow component of binding defined by resistance
to dissociation in the presence of an acid wash.

This conclusion is supported by two results: (a) the proportion
of NGF-receptor internalized is not affected by the concen-
tration of NGF present, and (b) in a model of binding and
internalization assuming equal rates of internalization for
occupied and unoccupied receptor, the experimental data fit
the curves determined from numerical integration of the rate
equations very well. Thus, it appears that the NGF receptor
is constitutively internalized. This is similar to the behavior
of transferrin receptor (38, 39). The rates of internalization
and of reappearance at the cell surface for NGF described
above do not differ substantially from the rates observed for
transferrin receptor (38). Similar plasmid mutants have also been
described for insulin (40). These results provide a relatively
simple model to explain binding of NGF to its receptor and
subsequent internalization of the complex.

Previously, Woodruff and Neet (1) performed an extensive
analysis of the binding characteristics of NGF-receptor. From
this analysis, they concluded that at least two forms of the
NGF-receptor were detectable after incubation of 125I-NGF at
37 °C. Our results agree with that interpretation. We observed
two kinetically distinguishable forms of NGF-receptor after
incubation at 37 °C. However, Woodruff and Neet (1) only
measured aggregate complex formation and, thus, were not
able to distinguish between two possible models for the origin
of the slow, high affinity NGF-receptor: (a) two independent
sites or (b) sequential formation of the second type of site
from the first type. By using a concentration of 125I-NGF
below the K_s of binding combined with a low concentration
of receptor such that the free NGF concentration was not
substantially reduced during the binding reaction, our exper-
iments were carried out under pseudo-first order conditions.
Thus, the data generated were analyzed without resorting to
fitting second order rate equations. We also designed exper-
iments to quantitate fast binding separate from slow or inter-
ialized receptors. This allowed us to distinguish between the
model for two independent receptors and the model for se-
quential formation of the second kinetically distinguishable
type of binding. Only the sequential model is consistent with
our data.

Association of NGF-receptor with cytoskeleton in the ab-
ence of NGF (2) is readily explained, if the receptor is
constitutively internalized. Thus, at any time, approximately
30% of NGF-receptor would be internal, while the rest is
present on the cell surface, even in the absence of NGF.
Triton extraction (2, 25-27) should routinely find a proportion
of NGF-receptor associated with the cytoskeletal extract of
cells, and the degree of association with the cytoskeleton could
be modified by pretreatment with wheat germ agglutinin (25).
It is even possible that the affinity of receptor when associated
with cytoskeletal proteins is different from the affinity of
receptor on the cell surface. However, measurement of binding
under these conditions is probably complicated by the need
to diffuse the ligand into the dense cytoskeletal extract, es-
pecially after treatment with wheat germ agglutinin which
agglutinates PC12 cells.3

Recent work (34) indicates that the difference between type
II (fast) NGF-receptor and type I (slow) receptor is the
location of the receptor in the cell. In this case, slow receptor
was shown to be associated with sequestration and internali-
ization while fast receptor was shown to be representative of
receptor free on the cell surface. Intercalation of receptors
was accomplished by agents which modulated the position of
receptor within the cell. The conclusions drawn here are in
agreement with the experiments described by Eveleth and
Bradshaw (34).

Bothwell et al. (29) reported on mutant PC12 cell sublines
which lacked the slow form of receptor and also failed to
differentiate in the presence of NGF. Although it is not clear
how many or which genes were mutated in these cells, it is
interesting to speculate that these cell sublines include a
mutation at the NGF-receptor gene which prevents signal
transduction and/or eliminates the signal to internalize the
NGF-receptor complex. NR18 cells, a specific subline of these
nonresponsive cells which fail to express any detectable recep-
tor, were transfected with the gene for the human NGF
receptor (30). After transfection, the NR18 cells regained the
ability to induce c-fos transcription after treatment with NGF.
Since both high and low affinity forms of receptor were
reported, these analyses still leave open the question of the
role of high affinity NGF-receptors. It is not clear that the
induction of c-fos could only be mediated by the high affinity
form of receptor.

It is possible that our failure to detect the presence of slow
receptor distinguishable from internalized receptor is due to
a difference in the strain of PC12 cells used by us compared
to the strains used by other investigators. However, even if
this is true, the slow form of NGF-receptor is not required for
neurite outgrowth in our strain of PC12 cells, since we can
readily observe neurite outgrowth at low concentrations of
NGF (Fig. 10). Thus, for our PC12 cells, the absence of slow
receptors does not preclude biological activity, although our
data do not address the issue of whether internalization is
required for biological activity. However, with the present
information in hand it is now more practical to address the
issue of the role of NGF internalization by NGF-receptor in
the biological activity of this growth factor. These data also
suggest another potential role for NGF-receptor, the ability
to move intact NGF across cells. The role of NGF-receptor
in the transcytosis of NGF is worthy of further investigation.

Since the rate of formation of slow binding is indistinguish-
able from the rate of internalization of 125I-NGF, since the
slow component of dissociation from PC12 cells is indistin-
guishable from the rate of externalization of NGF under
conditions described above (Fig. 8, and since some of the
properties of binding to slow receptor are consistent with
binding to a low affinity component rather than a high affinity
component, it is reasonable to hypothesize that the slow
binding has been confused with two properties of NGF bind-
ing: 1) internalization of the NGF-NGF-receptor complex,
and 2) the presence of very low affinity, very high capacity
(nonspecific) binding sites. This leaves a straightforward
interpretation of the binding properties of NGF. After ac-

3 S. Busser and D. Decker, unpublished observations.
counting for a potentially confusing amount of nonspecific binding and with internalization of the NGF receptor complex, all of the data are consistent with the presence of a single class of binding sites on the surface of PC12 cells. The biologically relevant sites have an affinity of approximately 0.5 nM, can be internalized by the cells, and require only a low level of occupancy, albeit for a long period of time, to initiate activation of the neurite outgrowth response.

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SUPPLEMENTARY MATERIAL
RELATIONSHIP AMONG TYPES OF NERVE GROWTH FACTOR RECEPTORS ON PC12 CELLS

Stephen Benzer, Douglas Becker and Patricia Ruppel

MATERIALS AND METHODS

Pathway of binding

The following two pathways were evaluated. Derivation of relevant equations is given in the Appendix.

Model I. Two-independent-receptor model

\[ \text{NGF} + R_2 \rightarrow \text{NGF-R}_2 \text{ (low affinity)} \]

Model II. Sequential formation (activation model)

\[ \text{NGF} + R_2 \rightarrow \text{NGF-R}_2 \rightarrow \text{NGF-R}_3 \]

Materials

Murine nerve growth factor was purified from mouse salivary glands according to the method described by M. G. Buxer. Further purification was achieved by affinity chromatography on Sepharose 4B and Protease inhibitors (Roche, Mannheim, Germany). The specific activity was determined using the bioassay described by M. G. Buxer. The NGF receptor was tested for biological activity and was indistinguishable from isolated NGF in a PC12 neurite outgrowth assay.

In the binding assay, NGF was incubated with cell suspensions at 4°C for 45 min. After the assay, cells were washed with medium containing 0.1 M sodium phosphate buffer (pH 7.4) and 1 M sodium chloride.
RESULTS

Results from two types of assays, one (radiochemically labeled and done by incubation in the presence of a high concentration of unlabeled NGF) and the other (using a high concentration of unlabeled NGF, as there is no experiment that would detect the presence of a high concentration of unlabeled NGF) for each system were obtained to evaluate the formation of specific binding and nonspecific binding and to evaluate the possibility of using a high concentration of unlabeled NGF in the assay. The data were analyzed using a computer program that took into account the possibility of high affinity sites being present in the assay. The results indicated that the binding of NGF to PC12 cells was not affected by the presence of unlabeled NGF. The binding of NGF to PC12 cells was not affected by the presence of unlabeled NGF.

Figure 1 shows a typical example of two types of assays for each system. One (radiochemically labeled and done by incubation in the presence of a high concentration of unlabeled NGF) and the other (using a high concentration of unlabeled NGF, as there is no experiment that would detect the presence of a high concentration of unlabeled NGF) for each system were obtained to evaluate the formation of specific binding and nonspecific binding and to evaluate the possibility of using a high concentration of unlabeled NGF in the assay. The data were analyzed using a computer program that took into account the possibility of high affinity sites being present in the assay. The results indicated that the binding of NGF to PC12 cells was not affected by the presence of unlabeled NGF. The binding of NGF to PC12 cells was not affected by the presence of unlabeled NGF.

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Figure 2 shows a typical example of two types of assays for each system. One (radiochemically labeled and done by incubation in the presence of a high concentration of unlabeled NGF) and the other (using a high concentration of unlabeled NGF, as there is no experiment that would detect the presence of a high concentration of unlabeled NGF) for each system were obtained to evaluate the formation of specific binding and nonspecific binding and to evaluate the possibility of using a high concentration of unlabeled NGF in the assay. The data were analyzed using a computer program that took into account the possibility of high affinity sites being present in the assay. The results indicated that the binding of NGF to PC12 cells was not affected by the presence of unlabeled NGF. The binding of NGF to PC12 cells was not affected by the presence of unlabeled NGF.

Figure 3 shows a typical example of two types of assays for each system. One (radiochemically labeled and done by incubation in the presence of a high concentration of unlabeled NGF) and the other (using a high concentration of unlabeled NGF, as there is no experiment that would detect the presence of a high concentration of unlabeled NGF) for each system were obtained to evaluate the formation of specific binding and nonspecific binding and to evaluate the possibility of using a high concentration of unlabeled NGF in the assay. The data were analyzed using a computer program that took into account the possibility of high affinity sites being present in the assay. The results indicated that the binding of NGF to PC12 cells was not affected by the presence of unlabeled NGF. The binding of NGF to PC12 cells was not affected by the presence of unlabeled NGF.
Forms of NGF-receptors

The results presented in this paper show that the presence of NGF receptor in the cell surface is an essential requirement for the survival of neurons in vitro. The presence of NGF receptor on the cell surface is determined by the concentration of NGF in the medium. The higher the concentration of NGF, the higher the number of NGF receptors on the cell surface.

The reaction can be described as:

\[ N + R \rightleftharpoons N\cdot R \]

The rate of association is given by the association constant, \( K_a \), and the rate of dissociation is given by the dissociation constant, \( K_d \). The equilibrium constant is given by the ratio of the forward rate constant to the reverse rate constant:

\[ K_e = \frac{K_a}{K_d} \]

The equilibrium constant is related to the dissociation constant by the equation:

\[ K_d = \frac{K_e}{K_a} \]

The reaction can be described as:

\[ N + R \rightleftharpoons N\cdot R \]

Under pseudo-first order conditions, the rate of dissociation is given by:

\[ \frac{dN}{dt} = -k_d N \]

Solving for the dissociation rate constant, we get:

\[ k_d = \frac{dN}{dt} \]

The dissociation constant is given by:

\[ K_d = \frac{k_d}{k_a} \]

The equilibrium constant is given by:

\[ K_e = \frac{K_a}{K_d} = \frac{k_a}{k_d} \]

Therefore, the ratio of NGF inside the cell as a proportion of the total cell-associated NGF is a constant determined by the ratio of the sum of \( k_a \) and \( k_d \).