Conversion of Nerve Growth Factor–Receptor Complexes to a Slowly Dissociating, Triton X-100 Insoluble State by Anti Nerve Growth Factor Antibodies†

Ronald D. Vale and Eric M. Shooter*

ABSTRACT: Two populations of nerve growth factor (NGF) receptors can be distinguished on PC12 cells and have been termed "fast" and "slow" receptors on the basis of their respective rates of dissociation. Since a slowly dissociating state of the NGF receptor could be produced by receptor clustering, we sought to examine the effects of artificially clustering NGF–receptor complexes by using anti-NGF antibodies. At an ^{125}I-NGF concentration of 500 pM, slow receptors account for 25% of the total NGF binding. However, if ^{125}I-NGF is first bound to the cell surface and an anti-NGF immunoglobulin G (IgG) is added subsequently, >90% of the total binding becomes slowly dissociating. Anti-NGF IgG also changes the proportion of Triton X-100 insoluble NGF binding from 10% to 50%, possibly reflecting an association of the NGF receptor with the cytoskeleton. The effects on NGF binding of low concentrations of anti-NGF IgG could be enhanced by the addition of goat anti-rabbit IgG. Neither F_{ab} fragments nor an anti-NGF monoclonal antibody affects NGF binding, indicating that the cross-linking capacity of anti-NGF IgG is required for its activity. Wheat germ agglutinin (WGA), a multivalent lectin, also converts NGF binding into a predominantly slowly dissociating, Triton X-100 insoluble state. Both WGA and anti-NGF IgG produce their effects on NGF binding at 37 and 4 °C and protect the ^{125}I-NGF–receptor complex from protease digestion. WGA and anti-NGF IgG act synergistically to convert NGF binding into a Triton X-100 insoluble form and affect NGF binding to the melanoma A875 cell line in a similar fashion as to PC12 cells. Since WGA and anti-NGF IgG share the potential capability of cross-linking NGF–receptor complexes, these results suggest that receptor clustering may play a role in the formation of a slowly dissociating, Triton X-100 insoluble state of the NGF receptor on PC12 and A875 cells.

Since its discovery over 25 years ago, nerve growth factor (a 26 000-dalton polypeptide) has remained the only purified agent whose importance in neuronal development has been clearly demonstrated (Thoenen & Barde, 1980). The actions of nerve growth factor (NGF) on the pheochromocytoma clonal cell line PC12 have been extensively studied in recent years as a model system for understanding neuronal differentiation (Greene & Trischler, 1976). NGF causes this cell line of neural crest origin to extend neurites and become electrically excitable.

Two distinct populations of cell-surface receptors for NGF have been identified on PC12 cells on the basis of their dissociation kinetics (Landreth & Shooter, 1980; Schechter & Bothwell, 1981). Upon the addition of excess unlabeled ligand, ^{125}I-NGF is released rapidly from one receptor subtype ($t_{1/2}$ of dissociation = 30 s) and slowly from another ($t_{1/2}$ of dissociation = 30 min). The two receptor species have been termed "fast" and "slow" by Schechter & Bothwell (1981) in reference to their dissociation properties. Slow receptors can be further distinguished from fast receptors by their resistance to degradation by trypsin (Landreth & Shooter, 1980; Schechter & Bothwell, 1981) and by their relative insolubility after Triton X-100 extraction (Schechter & Bothwell, 1981). Landreth & Shooter (1980) originally proposed that slow receptors may arise from fast receptors by a ligand-induced conversion process. Schechter & Bothwell (1981), on the other hand, suggested that fast and slow receptors are separate and independent entities. In an attempt to distinguish between such models, Vale & Shooter (1982) found that fast receptors could be converted into slow receptors by the lectin wheat germ agglutinin (WGA), indicating that the two receptors may be interrelated. The WGA-induced receptor conversion occurred within seconds, was largely independent of temperature, and was accompanied by an association of the NGF receptor with the Triton X-100 insoluble cytoskeleton, findings which have been independently observed by Grob and Bothwell (unpublished results). More recently, Buxser et al. (1983) have extended these observations to show that WGA also induces a receptor conversion in detergent-soluble membrane extracts and membrane extracts reconstituted into phospholipid vesicles.

Since WGA is a multivalent lectin, its ability to cross-link receptors may be important in producing its effects on the binding properties and Triton X-100 solubility of the NGF receptor. Since it has not been possible to produce a functionally monovalent derivative of WGA, as has been achieved with concanavalin A (Gunther et al., 1973), we decided to investigate how other clustering agents affect the binding properties of the NGF receptor. Schechter et al. (1979a) previously demonstrated that anti-insulin immunoglobulin G (IgG) increased the specific binding of ^{125}I-insulin to liver membranes by apparently converting a class of low-affinity receptors to high-affinity sites. The clustering of insulin receptors was important for this process, since monovalent F_{ab} fragments did not produce this effect. In the present study, we have investigated the effects of anti-NGF IgG on the binding properties of the NGF–receptor complex. Similar to the results previously reported with WGA (Vale & Shooter, 1982), anti-NGF IgG converter receptor-bound ^{125}I-NGF into a slowly dissociating and protease-resistant state. These antibodies also increased by severalfold the proportion of ^{125}I-NGF binding which is insoluble after Triton X-100 extraction. Conversion of NGF bound to fast receptors into a slowly

† From the Department of Neurobiology, Stanford University Medical Center, Stanford, California 94305. *Received May 2, 1983.* This work was supported by grants from the National Institutes of Health (NS 04270) and the American Cancer Society (BC 325). R.D.V. is a trainee in the Medical Scientist Training Program supported by a grant from the NIH (GM 07365).

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dissociating, Triton X-100 insoluble form by cross-linking agents such as WGA and anti-NGF IgG may be similar to a generation of slow from fast receptors through a process involving receptor clustering.

Materials and Methods

Materials. Wheat germ agglutinin was purchased from EY and Vector Laboratories. Goat anti-rabbit IgG was obtained from Cappel Laboratories. Papain (type III) and trypsin (type I) were obtained from the Sigma Chemical Co. Nerve growth factor was purified from adult male Swiss Webster mice as previously described (Burton et al., 1978). NGF was iodinated to a specific activity of between 50 and 90 cpm/pg by using a lactoperoxidase procedure (Sutter et al., 1979). The majority of ^{125}I-NGF binding assays were performed within 10 days of iodination.

Preparation of Anti-NGF IgG and F_{ab} Fragments. A purified IgG fraction was prepared from rabbit antisera to the β-NGF subunit or from preimmune sera by a previously published procedure involving diethylaminoethyl (DEAE) column chromatography (Reif, 1969). F_{ab} fragments were prepared from the IgG fraction by digestion with papain (Garvey et al., 1977). F_{ab} fragments were separated from F_c fragments and intact IgG by passing the mixture through a protein A–Sepharose CL 4-B column equilibrated with 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.4, and 150 mM NaCl. F_{ab} fragments were eluted in this same buffer, and F_c and IgG were eluted with 100 mM sodium acetate, pH 4. Monoclonal antibodies against NGF were obtained from a hybridoma cell line which has been previously described (Zimmerman et al., 1981). Serum-free hybridoma supernatants were collected and passed over a protein A–Sepharose CL 4-B column, and the column was washed with 10 column volumes of the equilibration buffer described above. Monoclonal IgG's were then eluted with 100 mM sodium acetate, pH 4, and 0.4-mL fractions were collected in tubes containing 0.15 mL of 0.5 M phosphate buffer, pH 7.5. Antibodies were then dialyzed against Dulbecco's phosphate-buffered saline (PBS). Protein concentrations of the antibodies were estimated by assuming an extinction coefficient of $E^{0.1\%}_{280nm} = 1.4$.

Cell Culture. PC12 cells were grown in 100-cm^2 Falcon dishes with Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum and 5% horse serum and were maintained at 37 °C in an atmosphere of 88% air–12% CO_2. The human melanoma A875 cell line (Fabricant et al., 1977a,b) was obtained from G. Todaro (NIH) and was grown in 75-cm^2 Falcon flasks in DME containing 10% fetal calf serum. Cells were passaged every 7 days.

^{125}I-NGF Binding Assay. Binding of ^{125}I-NGF to PC12 cells has been described in detail elsewhere (Vale & Shooter, 1982). Essentially, ^{125}I-NGF was added to PC12 cells (10^6 cells/mL) in suspension in Dulbecco's phosphate-buffered saline containing 1 mg/mL bovine serum albumin and glucose (binding buffer). Aliquots (100 μL) were then layered over 200 μL of 0.15 M sucrose in binding buffer in 400-μL microfuge tubes and centrifuged for 30 s at 10000*g* in a microfuge. Bound and free ^{125}I-NGF were separated by cutting the tubes above the cell pellets. To determine nonspecific binding, some samples were incubated with ^{125}I-NGF and a 500-fold excess of unlabeled NGF. Nonspecific binding accounted for <10% of the total binding. Nonspecific binding was subtracted from total binding to yield specific ^{125}I-NGF binding which is reported for all experiments. Slowly dissociating NGF binding was determined by adding 0.4 mL of cell sample containing ^{125}I-NGF to a 500-fold excess of unlabeled NGF for 30 min at 4 °C and then centrifuging 100-μL aliquots through a sucrose solution as described above. Nonspecific binding was subtracted from the pelleted radioactivity to yield slowly dissociating ^{125}I-NGF binding. Triton X-100 insoluble binding was determined by centrifuging 100-μL aliquots of PC12 cells and ^{125}I-NGF through 200 μL of 0.5% Triton X-100 in 0.3 M sucrose, 3 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.4, for 30 s at 10000*g* in a microfuge. Radioactivity associated with the Triton X-100 insoluble material which pelleted to the bottom of the tube had nonspecific binding (which also was centrifuged through this Triton X-100 solution) subtracted to yield Triton X-100 insoluble binding.

In preparation for the NGF binding assay, A875 cells were removed from the flask by incubating with (Ca^{2+}, Mg^{2+})-free PBS containing 1 mM ethylenediaminetetraacetic acid (EDTA) for 5 min at 37 °C. Cells were washed twice with binding buffer and collected by centrifugation at 500*g* for 5 min. The remainder of the binding assay was identical with the procedure described above for PC12 cells.

General Protocol for Testing the Effect of Antibodies on ^{125}I-NGF Binding. ^{125}I-NGF (at a concentration of approximately 500 pM unless noted) was added to 10^6 cells/mL in binding buffer for 30 min at 37 °C, by which time equilibrium binding is achieved (Landreth & Shooter, 1980; Vale & Shooter, 1982). The cell suspension was then centrifuged for 5 min at 500*g*, the supernatant removed, and the original volume of ^{125}I-NGF-free binding buffer added. Cells were then incubated for an additional 30 min at 37 °C in the presence or absence of antibody of F_{ab} fragment. The concentration of anti-NGF IgG used was 25 μg/mL unless specified. Total, nonspecific, slowly dissociating, and Triton X-100 insoluble binding were then determined as described previously. Nonspecific binding was determined with protocol identical with that of total binding and was not significantly affected by the presence of anti-NGF antibodies.

Results

Effects of Anti-NGF Antibodies on the Binding Properties of the NGF–Receptor Complex. Schecther et al. (1979a) previously documented that anti-insulin IgG incubated simultaneously with ^{125}I-insulin and liver membranes increased the specific binding of that ligand by 7–15-fold. In contrast to these findings, anti-insulin IgG decreased rather than increased ^{125}I-insulin binding to adipocytes (Schechter et al., 1979a). We have conducted similar experiments to determine the effects of anti-NGF IgG on ^{125}I-NGF binding to PC12 cells. When ^{125}I-NGF and anti-NGF IgG were mixed just prior to the addition of cells, ^{125}I-NGF binding could not be detected subsequently (data not shown). Monovalent F_{ab} fragments prepared from anti-NGF IgG and a monoclonal antibody against NGF (MC-β1) (Zimmerman et al., 1981) also decreased ^{125}I-NGF binding by 90% or greater.

The experimental protocol was then altered to investigate the effects of anti-NGF IgG on the ^{125}I-NGF–receptor complex. ^{125}I-NGF was first allowed to reach equilibrium with its receptors, and free ^{125}I-NGF was removed prior to the addition of the antibody. Being bivalent, anti-NGF antibodies have the potential to cluster NGF–receptor complexes and therefore could change the binding properties of these receptors. In the absence of antibodies, two populations of NGF receptors can be distinguished on the basis of their dissociation kinetics. At 4 °C, in the presence of excess unlabeled ligand, ^{125}I-NGF bound to fast receptors completely dissociates within 30 min while ligand remains bound to slow receptors in this period of time (Landreth & Shooter, 1980). Normally, at an ^{125}I-NGF concentration of 500 pM, slow receptors account

Table I: Effect of Antibodies on the Binding Properties of the ^{125}I-NGF–Receptor Complex[a]

treatment	total binding (fmol/10^6 cells)	slowly dissociating binding		Triton X-100 insoluble binding	
		fmol/10^6 cells	% of total	fmol/10^6 cells	% of total
control	139.7 ± 6.6	39.6 ± 2.0	28.4	11.8 ± 2.7	6.4
anti-NGF IgG	135.7 ± 3.8	118.2 ± 4.0	87.1	59.3 ± 9.6	43.7
anti-NGF F_{ab}	111.6 ± 2.7	39.9 ± 1.1	35.7	21.4 ± 6.8	19.2
monoclonal anti-NGF IgG	48.4 ± 5.9	31.8 ± 1.0	65.8	11.8 ± 1.7	24.4
preimmune IgG	138.3 ± 3.9	36.3 ± 1.7	26.2	13.6 ± 0.5	9.8

[a] The experimental procedure for measuring the effects of antibodies on ^{125}I-NGF already bound to PC12 cells is described under Materials and Methods. The following concentrations of antibodies were used: anti-NGF IgG, 25 μg/mL; anti-NGF F_{ab} fragments, 85 μg/mL; monoclonal (MC-β1) anti-NGF IgG, 85 μg/mL; preimmune IgG, 70 μg/mL. Results are expressed as the mean and standard deviation of triplicate determinations.

for approximately 25% of the total ^{125}I-NGF binding. Because of the removal of free ^{125}I-NGF prior to antibody addition, some of the ^{125}I-NGF bound to fast receptors dissociates (approximately 40%) while the majority of the ^{125}I-NGF on slow receptors remains bound. More rapid dissociation is not seen, because infinite dilution conditions are not employed and because dissociations of cell-bound radiolabeled ligand is greater in the presence of excess unlabeled ligand than under dilution conditions alone (Sutter et al., 1979).

As shown in Table I addition of anti-NGF IgG dramatically changed the dissociation kinetics and Triton X-100 solubility of the ^{125}I-NGF–receptor complex. Anti-NGF IgG increased the proportion of slowly dissociating binding from 28% to 87% and Triton X-100 insoluble binding from 6% to 44%. Total binding was not affected. The increase in slowly dissociating and Triton X-100 insoluble binding was not due to the formation of an insoluble antigen–antibody complex in solution, since no radioactivity was pelleted if only ^{125}I-NGF (with or without excess unlabeled NGF) and anti-NGF IgG were present in the reaction mixture (data not shown). Furthermore, Triton X-100 insoluble counts in the presence and absence of anti-NGF IgG pelleted at very low centrifugal forces (500*g* for 5 min), suggesting that the ^{125}I-NGF was associated with a large structure as the cell cytoskeleton. The alteration of NGF binding produced by anti-NGF IgG was not observed with an IgG fraction derived from preimmune sera or with univalent F_{ab} fragments derived from the anti-NGF IgG. Since F_{ab} fragments were ineffective, it appeared as though clustering of NGF–receptor complexes by intact IgG molecules may be important in the conversion of NGF binding to a slowly dissociating and Triton X-100 insoluble state.

Interestingly, the monoclonal (MC-β1) IgG behaved in a much different fashion than the polyclonal IgG (Table I). In contrast to the results obtained with polyclonal antibodies, monoclonal IgG did not convert fast receptors with bound ^{125}I-NGF into a slowly dissociating form and did not change the Triton X-100 solubility of NGF binding. However, in the presence of MC-β1 IgG, total binding decreased either because the antibody promoted the dissociation of ^{125}I-NGF from fast receptors or because it combined with ^{125}I-NGF after the dissociation of the ligand from fast receptors and thereby prevented its rebinding. Slow receptor binding was not significantly influenced by MC-β1 IgG. The different results obtained with polyclonal and monoclonal antibodies may be due to their relative effectiveness as cross-linking agents. Although bivalent, monoclonal antibodies tend to bind membrane-bound antigens univalently and are less capable of cross-linking these antigens than polyclonal antibodies (Mason & Williams, 1980). Furthermore, the maximum number of possible cross-linked receptors with monoclonal antibodies is two. Therefore, these results further argue that IgG-induced clustering of ligand–receptor complexes is important for converting fast receptors to a slow receptor state.

If clustering of NGF–receptor complexes by IgG is the important event in converting them to a slowly dissociating, Triton X-100 insoluble state, it may be possible to enhance these effects by promoting IgG-induced receptor clustering by the addition of a second antibody, a goat anti-rabbit IgG, which could cluster the cell-bound anti-NGF IgG molecules. Figure 1 shows an anti-NGF antibody concentration curve for the conversion of NGF binding to a slowly dissociating, Triton X-100 insoluble state in the presence or absence of a second goat anti-rabbit antibody. Total NGF binding was not affected by the presence of either or both antibodies. Anti-NGF IgG alone increased the amount of slowly dissociating binding with a maximal effect being achieved in most experiments at 25 μg/mL (Figure 1B). The conversion of NGF binding to a Triton X-100 insoluble form by anti-NGF IgG also showed a similar concentration dependence. However, while the ratio of slowly dissociating to total binding approached 1 at high antibody concentrations, the ratio of Triton X-100 insoluble to total binding plateaued at approximately 0.5.

If the goat anti-rabbit IgG was added subsequent to anti-NGF IgG, the dose–response of the effects of anti-NGF IgG on NGF binding was shifted to lower anti-NGF IgG concentrations. While goat anti-rabbit IgG potentiated the effects of anti-NGF IgG at submaximal doses, it did not alter the maximal level of slowly dissociating or Triton X-100 insoluble binding achieved in the presence of high concentrations of anti-NGF IgG. Goat anti-rabbit IgG increased the level of Triton X-100 insoluble binding in the absence of anti-NGF IgG for reasons which are unclear. The potentiation of the effects of anti-NGF IgG at submaximal concentrations by goat anti-rabbit IgG is consistent with the notion that antibody-induced clustering of NGF–receptor complexes produces an alteration of the receptor binding properties.

Time Course and Temperature Dependence of the Effects of Anti-NGF IgG. Figure 2 shows a time course of the conversion of NGF binding by anti-NGF IgG to a slowly dissociating and partially Triton X-100 insoluble state. The increase in slowly dissociating binding plateaued by 10–15 min, while the increase in Triton X-100 insoluble binding showed a more rapid time course and was complete by 5 min. Slowly dissociating binding and Triton X-100 insoluble bindings were nearly equal at early time points but were in a ratio of 2:1 by 15 min.

The effects of anti-NGF IgG on the NGF–receptor complex were largely independent of temperature (Table II). Similar results with respect to dissociating kinetics and Triton X-100 solubility were obtained whether the antibody incubation was performed at 37, 23, or 4 °C. These results argue that the effects of anti-NGF IgG occur at the cell surface and do not

Table II: Temperature Dependence of Antibody-Induced Slowly Dissociating, Triton X-100 Insoluble Binding[a]

temp (°C)	treatment	total binding (fmol/10^6 cells)	slowly dissociating binding		Triton X-100 insoluble binding	
			fmol/10^6 cells	% of total	fmol/10^6 cells	% of total
37	−IgG	113.4 ± 3.5	38.9 ± 1.1	34.2	8.7 ± 1.3	7.7
37	+IgG	95.3 ± 3.2	82.8 ± 2.2	86.9	36.9 ± 6.5	44.5
23	−IgG	97.6 ± 0.8	43.7 ± 2.7	44.8	13.0 ± 0.2	13.3
23	+IgG	99.7 ± 2.7	94.7 ± 2.5	95.0	52.7 ± 5.2	52.8
4	−IgG	99.3 ± 1.9	42.2 ± 1.7	42.4	20.1 ± 2.7	15.5
4	+IgG	85.5 ± 3.5	78.4 ± 1.0	91.7	48.2 ± 2.2	51.4

[a] The protocol for this experiment is described under Materials and Methods with the exception that the incubations with anti-NGF IgG were performed at either 37, 23, or 4 °C. Results are the mean and standard deviation of triplicate determinations.

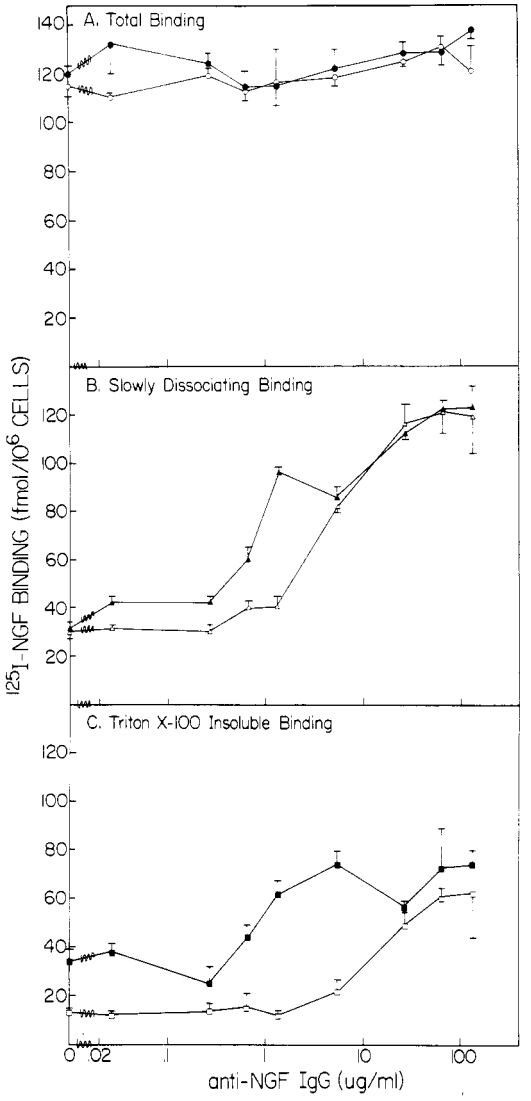

FIGURE 1: Enhancement of the effects of anti-NGF IgG by goat anti-rabbit IgG. ^{125}I-NGF was added to PC12 cells for 30 min at 37 °C. Cells were then centrifuged at 500*g* for 5 min, the supernatant was removed, and the cells were resuspended in binding buffer free of ^{125}I-NGF and incubated in the presence or absence of the indicated concentration of anti-NGF IgG for 30 min at 37 °C. Cell suspensions were then centrifuged as before, supernatants removed, and the cells resuspended in binding buffer in the absence (open symbols) or presence (closed symbols) of 63 μg/mL goat anti-rabbit IgG for 30 min at 37 °C, after which time binding was assayed. Total binding slowly dissociating binding, and Triton X-100 insoluble binding are shown in panels A, B, and C, respectively. Results are expressed as the mean and standard deviation of triplicate determinations.

involve internalization. Conversion of NGF receptors to a slowly dissociating, Triton X-100 insoluble state by WGA was also independent of temperature (Vale & Shooter, 1982).

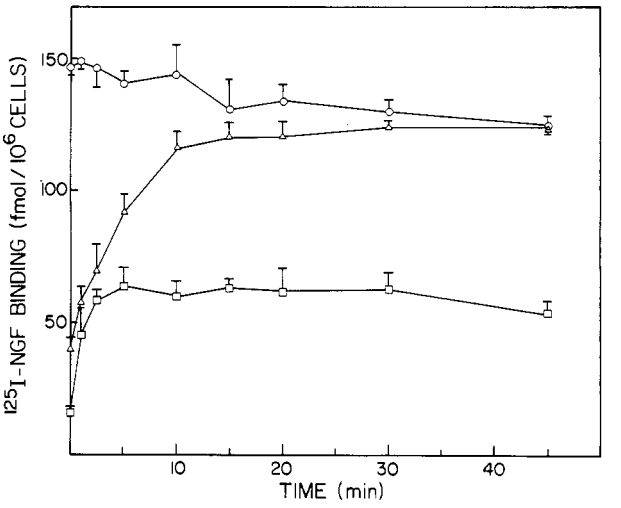

FIGURE 2: Time course of the conversion of ^{125}I-NGF binding to a slowly dissociating, Triton X-100 insoluble form by anti-NGF IgG. ^{125}I-NGF was added to PC12 cells for 30 min at 37 °C. Cells were then centrifuged at 500*g* for 5 min, supernatants were removed, and cells were resuspended in ^{125}I-NGF-free binding buffer. Total (○), slowly dissociating (△), and Triton X-100 insoluble (□) binding were measured as a function of time after the addition of anti-NGF IgG (25 μg/mL). Values represent the mean and standard deviation of triplicate determinations.

Kinetic Properties and Proteolytic Sensitivity of Antibody-Induced Slow Receptors. Figure 3 shows the dissociation kinetics of slowly dissociating receptors from antibody-treated and untreated cells. Antibody-treated cells demonstrated a significantly slower rate of dissociation than control cells after the addition of excess unlabeled NGF. The initial $t_{1/2}$ values of dissociation for control and antibody-induced slow receptors were 30 and 130 min, respectively. In these experiments, control cells demonstrated a somewhat slower dissociating component at longer time points which was very likely due to the release of internalized ^{125}I-NGF. Radioactivity released from cells within 30 min is almost all trichloroacetic acid (Cl_3CCOOH) precipitable, while between 30 and 60 min only 50% of the released radioactive counts are Cl_3CCOOH precipitable (R. D. Vale and E. M. Shooter, unpublished results). These results suggest that control and antibody-induced slow receptors may not be identical.

Slow receptors can also be distinguished from fast receptors by their resistance to proteolytic degradation by high concentrations of trypsin (Landreth & Shooter, 1980). Slow receptors induced by anti-NGF antibodies were also largely protected from proteolytic attack as seen in Table III. Anti-NGF IgG increased the proportion of ^{125}I-NGF binding which was resistant to trypsin degradation from 20% to 90%. Papain was also tested in the hope of generating F_{ab} fragments of bound IgG, and similar results to the ones described for trypsin were obtained. The binding which remained after

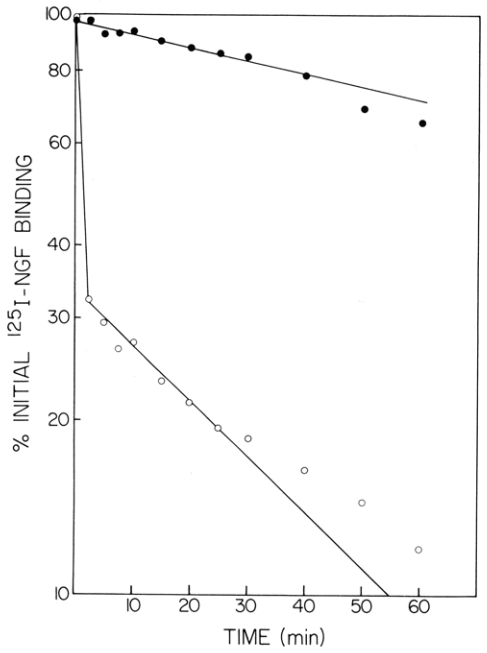

FIGURE 3: Dissociation kinetics of slowly dissociating ^{125}I-NGF binding in the presence and absence of anti-NGF IgG. ^{125}I-NGF was added to PC12 cells for 30 min at 37 °C. Cells were then centrifuged at 500*g* for 5 min, the supernatants were removed, and cells were resuspended in ^{125}I-NGF-free binding buffer. Half of the cells then received anti-NGF IgG (25 μg/mL) for an additional 30 min. Unlabeled NGF (500 nM) was then added, and dissociation of ^{125}I-NGF from slowly dissociating sites was measured at 37 °C from antibody-treated (●) and untreated (○) cells. Triplicate determinations were made at each time point. Results presented here have been averaged from two experiments and are expressed as the percent of the initial total binding before dissociation (149.8 fmol/10^6 untreated cells; 144.5 fmol/10^6 antibody-treated cells). The lines through the data points represent the initial rates of dissociation calculated by linear regression from the first eight time measurements.

Table III: Susceptibility of Antibody-Induced Slowly Dissociating Binding to Protease Digestion[a]

protease treatment	−IgG total binding	−IgG slowly dissociating binding	+IgG total binding	+IgG slowly dissociating binding
none	113.4 ± 3.5	38.9 ± 1.1	95.3 ± 3.2	82.8 ± 2.2
trypsin (0.5 mg/mL)	30.5 ± 0.5	29.1 ± 0.8	79.3 ± 3.0	73.4 ± 2.1
papain (1.0 mg/mL)	24.0 ± 0.6	21.5 ± 0.4	65.7 ± 1.5	57.7 ± 1.2

[a] Anti-NGF IgG was added to PC12 cells as described under Materials and Methods. Cells were then incubated with the indicated concentration of trypsin or papain for 20 min at 37 °C. Total binding slowly dissociating binding were then determined. Values are given as femtomoles bound per 10^6 cells. Results are expressed as the mean and standard deviation of triplicate determinations.

trypsin or papain treatment was >90% slowly dissociating, indicating that these proteolytic enzymes selectively removed ^{125}I-NGF bound to fast receptors. Identical results were obtained if the entire experiment was performed at 4 °C instead of 37 °C, suggesting that proteolytic protection is not the result of internalization (data not shown). It is not known whether anti-NGF IgG bound to the cell surface is also resistant to proteolysis.

Effects of WGA and Anti-NGF IgG on NGF Binding to PC12 and A875 Cells. Both WGA and anti-NGF IgG can

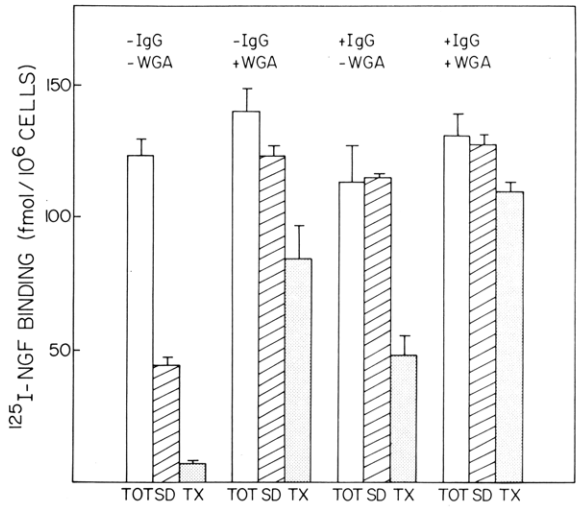

FIGURE 4: Effects of WGA addition on ^{125}I-NGF binding properties for cells treated with or without anti-NGF IgG. ^{125}I-NGF and anti-NGF antibodies were added to PC12 cells as described under Materials and Methods. Cells were subsequently incubated with or without WGA (50 μg/mL) for 15 min at 37 °C. Total binding (TOT), slowly dissociating (SD) binding, and Triton X-100 insoluble (TX) binding were determined at this time. Histograms are the mean and standard deviation of triplicate determinations.

change the Triton X-100 solubility and dissociation kinetics of the NGF–receptor complex. In the presence of either one of these agents, >90% of the cell-bound ^{125}I-NGF becomes slowly dissociating. However, while the maximal level of Triton X-100 insoluble binding in the presence of anti-NGF IgG is 50%, between 70% and 100% of the total NGF binding is Triton X-100 insoluble after WGA treatment (Vale & Shooter, 1982). The reason for this difference is not clear. Figure 4 shows that anti-NGF IgG and WGA act synergistically in increasing the proportion of NGF binding which is resistant to detergent solubilization. Antibodies increased the proportion of Triton X-100 insoluble binding to total binding from 5% to 35%. If WGA was subsequently added, Triton X-100 insoluble binding increased additionally from 35% to 88%. Therefore, the antibody-induced slowly dissociating receptors which were Triton X-100 soluble could still be converted to a Triton X-100 insoluble form by WGA. Since the binding site of the NGF receptor is occupied by the ligand and a IgG molecule WGA most likely produces its effects by binding to a region of the NGF receptor distinct from the ligand binding site. Alternatively, WGA could interact with another membrane protein closely associated with the receptor.

Fabricant et al. (1977a,b) demonstrated the presence of NGF receptors on the melanoma cell line A875. This cell line has 6–10 times the number of NGF receptors per cell than the PC12 cell line (Herrup & Thoenen, 1979). Figure 5 shows that WGA and anti-NGF IgG produced similar effects on NGF receptors on the A875 cell line as on PC12 cells. Slow receptors normally account for only 4–5% of the total binding on A875 cells as compared with 20–30% on PC12 cells. However, since the total receptor number is greater on the A875 cells, the absolute number of slow receptors per cell may be similar in the two cell types. As seen in this figure, both WGA and NGF antibodies increase by severalfold the proportion of slowly dissociating and Triton X-100 insoluble binding on A875 cells. As with PC12 cells, the amounts of slowly dissociating and detergent-insoluble binding were comparable on WGA-treated cells, while Triton X-100 insoluble binding was approximately half of the slowly dissociating binding for anti-NGF IgG treated cells. These results suggest that NGF receptors on PC12 and A875 cells may

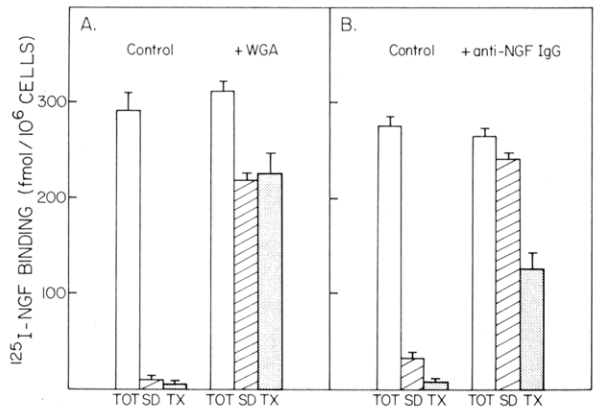

FIGURE 5: Effects of WGA and anti-NGF IgG on ^{125}I-NGF binding to A875 melanoma cells. (A) ^{125}I-NGF was added to 0.75 × 10^6 cells/mL for 30 min at 37 °C. WGA (50 μg/mL) was then added for 30 min at 37 °C, and total binding (TOT), slowly dissociating binding (SD), and Triton X-100 insoluble (TX) binding were determined. (B) ^{125}I-NGF and anti-NGF IgG were added to A875 cells as described under Materials and Methods and total (TOT) binding, slowly dissociating (SD) binding, and Triton X-100 insoluble (TX) binding were determined. Results in panels A and B are from independently conducted experiments. Bars represent the mean and standard deviation of triplicate determinations.

share many structural and functional features.

Discussion

As has been observed with a variety of hormone receptors (Weichman & Notides, 1977; Corin & Donner, 1982; Donner et al., 1980), NGF receptors display heterogeneous kinetic states. It is not known whether this heterogeneity is the result of two distinct receptor populations or due to conformational changes occurring in a single receptor molecule. In attempting to answer such question, we have identified agents which can convert one receptor subtype with rapidly dissociating properties into a slowly dissociating species. The change in kinetic state is accompanied by alterations in the proteolytic sensitivity and Triton X-100 solubility of the NGF receptor. The two agents (anti-NGF IgG and WGA) which produce these effects share in common a potential capability of clustering receptors. The receptor conversion induced by anti-NGF IgG and WGA may be similar to a ligand-induced receptor conversion process which has been proposed by Landreth & Shooter (1980).

The generation of slowly dissociating NGF–receptor complexes by anti-NGF IgG requires antibody cross-linking, since monovalent F_{ab} fragments or functionally monovalent MC-β1 monoclonal antibodies were not effective. Furthermore, enhanced cross-linking by goat anti-rabbit antibodies increased the potency of anti-NGF IgG. Cross bridging of two or more NGF–receptor complexes by antibodies in itself could account for the formation of slowly dissociating binding. In order for ^{125}I-NGF bound to such a complex to be released into solution, it must dissociate from two receptors rather than one. If only one NGF–receptor complex dissociates, the ligand will nonetheless remain at the cell surface since the other arm of the antibody to which it is attached is bound to another NGF–receptor complex. The close proximity of partially released ligand to the cell surface may also enable it to readily rebind to a receptor molecule. However, it should be noted that the effects we have seen with anti-NGF IgG have not been observed in other hormone systems, as Schechter et al. (1978) have reported that anti-EGF antibodies did not decrease the dissociation rate of ^{125}I-EGF bound to human fibroblasts.

The generation of slowly dissociating binding by cross-linking with anti-NGF IgG may be similar to a conversion of fast to slow receptors induced by NGF itself. NGF consists of two identical 13 000-dalton monomers joined by noncovalent forces (Angeletti & Bradshaw, 1971) and is therefore potentially divalent with respect to receptor binding. The binding of a divalent ligand to a homogeneous population of receptors could produce heterogeneous binding kinetics as has been proposed by DeLisi & Chabay (1979). NGF bound univalently would dissociate with rapid kinetics, while NGF bound divalently to two receptors would dissociate with slow kinetics for the reasons discussed above. Furthermore, this hypothesis could account for the curvilinear Scatchard plots and enhanced dissociation kinetics with unlabeled ligand which have been observed for NGF binding (Sutter et al., 1979). Alternatively, fast and slow NGF receptors could (1) be independent receptor molecules, (2) be related to one another by a change in the tertiary configuration of a single receptor or by the distribution of a single receptor molecule in different phospholipid domains, or (3) be generated from one another by an association of the receptor with a membrane protein which modulates receptor affinity (Jacobs & Guatrecasas, 1976). Current experimental data do not allow one to distinguish between these four models.

The relationship between the normal slow receptor and the antibody-induced slow receptor is uncertain. Although both types are classified as slowly dissociating binding, the off rate of ^{125}I-NGF from its receptor in the presence of antibody is 4-fold slower than that for the normal slow receptor. However, both types of slowly dissociating binding are similar in certain respects, as both share in common a resistance to proteolytic digestion and solubilization by Triton X-100. These effects were observed at 4 °C as well as 37 °C, indicating that they are not due to internalization.

These studies with anti-NGF IgG and WGA show that the change in the proportion of slowly dissociating binding was accompanied by an increase in the resistance of ^{125}I-NGF binding to Triton X-100 solubilization. Block & Bothwell (1983) also observed that fusion of PC12 cell-surface membranes to 3T3 cells results in a conversion of the transplanted NGF receptors to a slowly dissociating state, along with a corresponding increase in the Triton X-100 insolubility of NGF binding. It has been proposed that the resistance of ^{125}I-NGF binding to Triton X-100 extraction may reflect an association of the ligand–receptor complex to the cytoskeleton (Schechter & Bothwell, 1981; Vale & Shooter, 1982). An actin-containing matrix has been found in association with plasma membranes of various cell types (Moore et al., 1978; Mescher et al., 1981), and certain plasma membrane proteins were found attached to this structure after detergent solubilization (Ben-Ze'ev et al., 1979).

In some instances, the clustering of cell-surface proteins can induce their attachment to the cytoskeleton. Con A causes an interaction of two platelet cell-surface glycoproteins with cytoskeletal elements (Painter & Ginsberg, 1982). Also, IgG or $F_{ab}'2$ directed against lymphocyte surface immunoglobulins induces these molecules to associate with a detergent-insoluble cytoskeletal matrix (Flanagan & Koch, 1978; Woda & McFadden, 1982), and analogous to our results with anti-NGF IgG, cross-linking was important for this effect since F_{ab} fragments did not alter the detergent solubility of these proteins. So far, the relationship between slowly dissociating binding and cytoskeletal attachment is correlative, and there is no evidence of a causal event integrally relating the two together.

In summary, receptor clustering may be involved in a conversion of fast to slow receptors, a process which could involve the participation of cytoskeletal elements. Exogenous

agents which potentially cluster NGF receptors such as WGA or anti-NGF antibodies produce a receptor conversion which in some respects may reflect a naturally occurring conversion process. Receptor clustering may also be involved in the mechanism of action of hormones as has been suggested by experiments involving the cross-linking of epidermal growth factor receptors (Schechter et al., 1979b; Schreiber et al., 1983) and insulin receptors (Kahn et al., 1978; Cuatrecasas & Tell, 1973). It also would be of interest to identify substances which could block conversion of fast to slow receptors or shift slow receptor binding to a rapidly dissociating state. It is hoped that through these approaches one will learn more of the interrelationships and functions of the two NGF receptor populations on PC12 and A875 cells.

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