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Alteration of Binding Properties and Cytoskeletal Attachment of Nerve Growth Factor Receptors in PC12 Cells by Wheat Germ Agglutinin

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ABSTRACT Incubation of PC12 cells preloaded with ^{125}I-nerve growth factor (NGF) reveals rapidly and slowly dissociating binding components indicative of a heterogeneous population of receptors. If the cells are previously exposed to wheat germ agglutinin (WGA) for 30 min, NGF now binds to an apparently homogeneous receptor population which exhibit slow dissociation kinetics. Total binding is also reduced by 50%. If WGA is added subsequent to ^{125}I-NGF, total binding is not diminished, but rapidly dissociating receptors occupied with NGF are all converted to the slowly dissociating form. This conversion of receptors occurs rapidly, reaching completion within 2 min at 37° or 4°C, and is unaffected by metabolic energy poisons, suggesting that WGA-induced slowly dissociating receptors are not the product of internalization. The effects of the lectin are blocked by the sugar *N*-acetyl-D-glucosamine, and the lectin-induced slowly dissociating receptors are converted back to rapidly dissociating receptors by addition of this same sugar. WGA also affects the association of the NGF receptor with the Triton X-100 cytoskeleton. >90% of bound ^{125}I-NGF becomes associated with Triton X-100 insoluble cytoskeletons in the presence of the lectin, compared with <20% before lectin addition. Cytoskeleton association of the NGF receptor by WGA shows similar kinetics as the conversion of rapidly to slowly dissociating receptors. This interaction may be involved in the alteration of NGF-receptor binding properties produced by this lectin.

Nerve growth factor (NGF) is a polypeptide hormone which is involved in the development and maintenance of tissues derived from neural crest including the sympathetic nervous system (1). The development of a clonally derived cell line from a rat pheochromocytoma, PC12, which responds to NGF by acquiring some properties typical of sympathetic neurons, has been very useful for the study of the mechanism of action of NGF. Addition of NGF to these cells results in the cessation of cell division and extension of neuritic processes (2).

Like other polypeptide hormones, NGF binds to specific cell surface receptors on PC12 cells. Dissociation kinetics of cell-bound NGF reveal two distinct components (3). Such complex kinetics are incompatible with a homogeneous population of noninteracting receptors. Hormones, such as NGF (4) and insulin (5), also demonstrate curvilinear Scatchard plots which are indicative of complex binding interactions. A variety of models have been evoked in attempts to explain this heterogeneity of binding. Such models include a homogeneous population of receptors with negatively cooperative interactions (6), multiple and independent receptor sites (7), or modulation of receptor affinity by effector proteins in the membrane (8). Landreth and Schooter (3) have suggested that a ligand-induced conversion of low to high affinity receptors occurs on PC12 cells, a conversion which theoretically could be modulated by an effector protein in the membrane as described in the mobile receptor hypothesis of Jacobs and Cuatrecasas (8). On the other hand, Schechter and Bothwell (9) have concluded that NGF binds to two preexisting and noninteracting receptor populations on PC12 cells, receptors which differ in their attachment to the cytoskeleton.

To test whether an effector molecule modulating receptor affinity exists, agents have been sought which might inhibit, enhance or mimic its action and thereby alter the ratio of the two types of NGF receptors. In this study, we examine the effect of lectins, proteins which bind specific carbohydrate moieties, on NGF binding to PC12 cells. The results demonstrate that the lectin, wheat germ agglutinin (WGA), can convert a rapidly dissociating population of NGF receptors into a slowly dissociating form. This alteration in binding properties of the receptor is accompanied by a rapid association

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of the NGF receptor complex with the Triton X-100 insoluble cytoskeleton and raises the possibility that the cytoskeleton or a cytoskeleton-associated protein may act as an effector molecule in modulating receptor properties.

MATERIALS AND METHODS

Materials

Lectins were purchased from either EY Laboratories Inc. (San Mateo, CA) or Vector Laboratories, Inc. (Burlingame, CA). Succinylinate concanavalin A (succinylinate Con A) was prepared as described by Gunther et al. (10). NGF (the β subunit) was prepared as described previously (11). NGF was iodinated by a lactoperoxidase technique to a specific activity of 50–70 cpm/pg (4) and was used within 3 wk.

Cell Culture

PC12 cells, originally isolated by Green and Tischler (2), were cultured in 100-cm^2 Falcon dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) with Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum and 5% horse serum and were maintained at 37°C at an atmosphere of 88% air, 12% CO_2. Cells were passaged every 7 d.

^{125}I-NGF Binding Assay

PC12 cells were washed twice on the dish with Dulbecco's phosphate-buffered saline containing 1 mg/ml each of glucose and bovine serum albumin (binding buffer). Cells were removed from the dish by trituration, counted using a hemocytometer and resuspended to a final concentration of 10^6 cells/ml in binding buffer. Binding of ^{125}I-NGF to PC12 cells was performed essentially as described by Landreth and Shooter (3). ^{125}I-NGF was incubated with PC12 cells for a given time. Aliquots (100 μl) were layered >200 μl of 0.15 M sucrose in binding buffer in 400-μl microfuge tubes. To separate bound from free ^{125}I-NGF, cells were centrifuged for 30 s at 10,000 *g* in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). Microfuge tubes were frozen in an ethanol/dry ice bath, cut above the pellets, and both cell pellets and supernatants counted in a Beckman gamma counter (Beckman Instruments, Inc.) Nonspecific binding is defined as radioactivity associated with the cell pellet in the presence of excess unlabeled hormone added at the beginning of the incubation. Nonspecific binding from both WGA-treated and -untreated cells accounted for <10% of the total binding. Only specific ^{125}I-NGF binding is reported in the experiments described here.

The proportion of slowly dissociating receptors was determined by the following method. After incubating PC12 cells with ^{125}I-NGF for a given period of time, 0.4 ml of cells were added to a 500–1,000-fold excess of unlabeled NGF at 4°C for 30 min. During this period of time, ^{125}I-NGF dissociates from the rapidly dissociating population of receptors, while NGF attached to slowly dissociating receptors remains bound at this temperature (3). To assay residual binding, 100-μl aliquots were centrifuged through 0.15 M sucrose as described above. Nonspecific binding was subtracted from the pelleted radioactivity to yield slowly dissociating binding. Rapidly dissociating binding was determined by subtracting slowly dissociating counts from the total specific binding.

Triton X-100 Insoluble ^{125}I-NGF Binding (Cytoskeleton-associated Binding)

^{125}I-NGF was incubated with cells for a certain period of time. After this incubation, 100-μl aliquots were layered >0.5% Triton X-100 in 0.3 M sucrose, 3 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4. This Triton X-100 containing buffer was used by Ben Ze'ev et al. (12) and Schechter and Bothwell (9) to solubilize membranes and prepare cytoskeletal elements. Microfuge tubes were centrifuged, cut, and counted as described for the binding assay. The radioactivity associated with the Triton X-100 insoluble material which pelleted to the bottom of the microfuge tube had nonspecific binding subtracted to yield Triton X-100 insoluble NGF binding.

RESULTS

Effect of Various Lectins on NGF Binding

The effects on total ^{125}I-NGF binding to PC12 cells of a 30-min preincubation with nine different lectins is shown in Table I. Of the lectins used, only WGA produced a significant inhibition (55%) of NGF binding. Concanavalin A (Con A) which has been reported to substantially reduce NGF binding to rabbit sympathetic ganglia (13), only produced a small inhibition of binding to PC12 cells. WGA reduced binding to both PC12 and sympathetic ganglion cells.

TABLE I

Effect of Preincubation of PC12 Cells with Lectins on Subsequent ^{125}I-NGF Binding and Dissociation

Lectin	Total binding	Slowly dissociating binding	
	pg ^{125}I-NGF/ 10^5 cells	*pg ^{125}I-NGF/ 10^5 cells*	*% Total*
None	204.2 ± 15.1	40.3 ± 2.0	19.7
Ricin Communis agglutinin I	195.9 ± 6.0	48.6 ± 2.6	24.8
Limulus Polyphemus agglutinin	200.9 ± 4.7	44.5 ± 0.3	22.2
Peanut agglutinin	208.1 ± 11.9	46.4 ± 2.6	22.3
Ulex Europeus agglutinin I	165.6 ± 4.2	41.8 ± 1.5	25.3
Soybean agglutinin	189.7 ± 0.5	48.0 ± 3.2	25.3
Con A	163.4 ± 4.1	63.2 ± 1.7	38.7
Succinylated Con A	185.0 ± 36.5	24.0 ± 0.8	13.0
Dolichos Biflorus agglutinin	189.5 ± 3.4	38.4 ± 3.2	20.3
WGA	95.1 ± 8.4	82.3 ± 3.0	86.5

PC12 cells were incubated with the indicated lectin (50 μg/ml) for 30 min at 37°C. ^{125}I-NGF (250 pM) was then added, and both total and slowly dissociating binding were determined as described in Materials and Methods. The data is the mean and standard deviation of triplicate samples.

Effect of WGA on NGF Binding

Previous work has demonstrated that NGF binds to a heterogeneous population of receptors which differ in their dissociation kinetics (3, 9). At 37°C and in the presence of excess unlabeled NGF, ^{125}I-NGF dissociates from one receptor population with rapid kinetics ($t_{1/2}$ = 30 s) and from another population of receptors much more slowly ($t_{1/2}$ = 30 min). These receptors correspond to the "Fast" and "Slow" receptors of Schechter and Bothwell (9). On the other hand, at 4°C NGF remains bound to the slowly dissociating receptors, while it can be completely removed from rapidly dissociating receptors within 15 min. Thus, by incubating cells which have reached equilibrium binding with a large excess of unlabeled NGF for 30 min at 4°C, it is possible to selectively dissociate ^{125}I-NGA bound to rapidly dissociating receptors.

This dissociation protocol has been used to examine whether WGA affected NGF binding preferentially to slowly or rapidly dissociating receptors. As shown in Fig. 1, in the absence of WGA, NGF bound to rapidly dissociating receptors within a few minutes (maximal association time is ~2 min [3]), while binding to slowly dissociating receptors required ~20 min to reach equilibrium. This data is in qualitative agreement with that of Schechter and Bothwell (9). When cells were treated with WGA for 60 min, almost complete inhibition of binding to rapidly dissociating receptors was observed. The majority of the total NGF binding (90%) was slowly dissociating in the presence of WGA compared with 45% in the absence of the lectin. Preincubation of the PC12 cells with WGA for times between 5 and 60 min produced quantitatively similar results (data not shown). NGF binding in the presence of WGA showed approximately the same rate of appearance as binding to slowly dissociating receptors in control cells.

Although the slowly dissociating binding from control and WGA-treated cells were quantitatively similar in the experiment in Fig. 1, this was only a function of the relatively low

^{125}I-NGF concentration. At higher ^{125}I-NGF concentrations, the slowly dissociating binding from WGA-treated cells exceeded that from control cells (Table I). WGA produced a consistent decrease (~50%) in NGF binding over a wide range of NGF concentrations, and only at low ^{125}I-NGF concentrations where slowly dissociating binding accounted for 40–50% of the total binding (e.g., see Fig. 6) were the absolute amounts of slowly dissociating binding from control and WGA-treated cells equivalent. Scatchard analysis indicated that the 50% decrease in binding produced by WGA was due to a decrease in receptor number with little change in affinity (data not shown). However the above findings indicate that the decrease in receptors is not due to a selective block of preexisting rapidly dissociating binding sites. The population of receptors which remain after WGA treatment demonstrate mostly homogeneous slow dissociation kinetics as opposed to the heterogeneous kinetics of NGF receptors on control cells.

The data in Table I demonstrates that lectins that did not significantly affect total binding also did not change the ratio of slowly to rapidly dissociating binding. The possible exception is Con A, which, while not producing a large reduction in total binding, increased the proportion of slowly dissociating binding. It is interesting to note that a succinylated derivative of Con A, which is a dimer rather than a tetramer like Con A, did not produce this effect. Succinylated Con A has been shown to have the same binding affinity as the native protein for α-methyl-mannoside residues, but has markedly reduced cross-linking abilities (10).

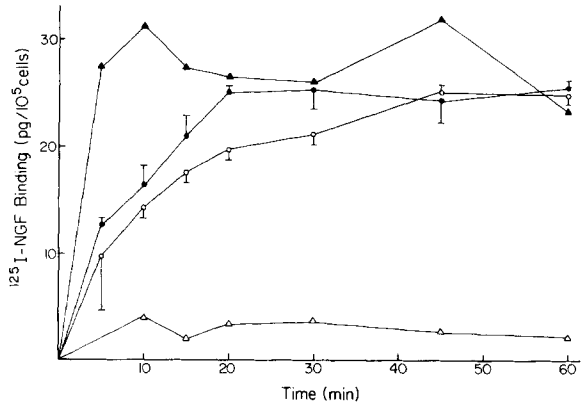

FIGURE 1 Effects of WGA on rapidly and slowly dissociating NGF binding. PC12 cells were incubated with or without 75 μg/ml WGA for 60 min at 37°C. ^{125}I-NGF (100 pM) was added, and at the indicated times, slowly dissociating binding (●, control; ○, WGA-treated) and rapidly dissociating binding (▲, control; △, WGA-treated) were determined as described in Materials and Methods. Values of this representative experiment are the mean of triplicate determinations and error bars indicate standard deviations.

The effect of WGA on NGF binding depended on the order of addition of WGA and ^{125}I-NGF. If WGA was added after NGF had reached binding equilibrium, no inhibition of total binding was observed (Table II); however, 90% of the total binding was in a slowly dissociating state. Therefore, WGA acts to convert rapidly dissociating receptors into a slowly dissociating form. Lectins which did not produce an inhibition of total NGF binding when preincubated with PC12 cells also did not convert rapidly dissociating binding to a slowly dissociable form when added subsequent to ^{125}I-NGF. Con A again had an effect less than that of WGA, while succinylated Con A behaved like the other lectins.

N-Acetyl-D-glucosamine, the sugar with the highest affinity for WGA, prevented the inhibition of NGF binding resulting from a 30-min preincubation with WGA. Fig. 2*A* shows that WGA produced close to a maximal decrease in total NGF binding at 50 μg/ml. The presence of 100 mM *N*-acetyl-D-glucosamine prevented this inhibition of binding at all WGA concentrations. The sugar by itself did not alter NGF binding. Furthermore, *N*-acetyl-D-glucosamine blocked the conversion of rapidly to slowly dissociating receptors by WGA. The data in Fig. 2*B* shows that WGA concentrations of 25 μg/ml or more produced complete conversion of receptors, while the presence of *N*-acetyl-D-glucosamine in the medium essentially eliminated this effect. As indicated by the data at 100 μg/ml WGA (Fig. 2*A* and *B*), all the binding in the presence of WGA was slowly dissociating binding, whereas this was not true when the sugar was present simultaneously.

NGF does not contain any glycosyl residues and is therefore an unlikely candidate to interact with a lectin (Bamburg, J. R., and E. M. Shooter, unpublished observations). Two experiments confirmed that WGA did not produce its effects on NGF binding by interacting directly with NGF. In one experiment, ^{125}I-NGF was found to associate to a similar extent with Sepharose 4B or with WGA conjugated to Sepharose 4B, and the bound NGF accounted for <10% of the total in both instances (data not shown). In addition, the continuous presence of WGA is not necessary to affect NGF binding. If cells, preincubated with WGA, were centrifuged and washed free of unbound WGA before addition of ^{125}I-NGF, a similar inhibition of binding was observed as when WGA was present continuously in the medium (data not shown).

TABLE II

Effect of Lectins on the Binding, Dissociation, and Triton X-100 Solubility of ^{125}I-NGF Previously Bound to PC12 Cells

Lectin	Total binding	Slowly dissociating binding		Triton X-100 insoluble binding	
	pg ^{125}I-NGF/10^5 cells	*pg ^{125}I-NGF/10^5 cells*	*% Total*	*pg ^{125}I-NGF/10^5 cells*	*% Total*
None	218.3 ± 12.2	52.8 ± 0.1	24.2	22.1 ± 1.8	10.1
Ricin Communis agglutinin I	244.2 ± 3.4	56.5 ± 0.3	23.1	23.7 ± 1.8	11.7
Limulus Polyphemus agglutinin	237.5 ± 1.7	66.7 ± 2.7	28.1	53.1 ± 12.8	22.3
Peanut agglutinin	207.9 ± 1.5	57.4 ± 4.9	27.6	31.4 ± 5.5	15.1
Ulex Europeus agglutinin I	207.7 ± 14.8	61.2 ± 1.8	29.4	49.3 ± 2.5	23.7
Soybean agglutinin	207.5 ± 12.2	69.9 ± 5.8	33.6	22.8 ± 10.9	11.0
Con A	230.8 ± 21.4	93.8 ± 0.6	40.6	109.5 ± 2.2	47.4
Succinylated Con A	220.4 ± 7.2	63.5 ± 4.8	28.8	35.3 ± 8.5	16.0
Dolichos Biflorus agglutinin	212.2 ± 3.3	59.6 ± 6.8	28.1	34.3 ± 4.6	16.2
WGA	250.2 ± 16.7	229.8 ± 12.1	91.9	224.0 ± 0.9	89.5

PC12 cells were incubated with ^{125}I-NGF (450 pM). After 30 min at 37°C, the indicated lectin was added at a concentration of 50 μg/ml. After an additional 30-min incubation, cells were assayed for total, slowly dissociating, and Triton × = 100 insoluble binding. The data are the mean and standard deviation of triplicate samples.

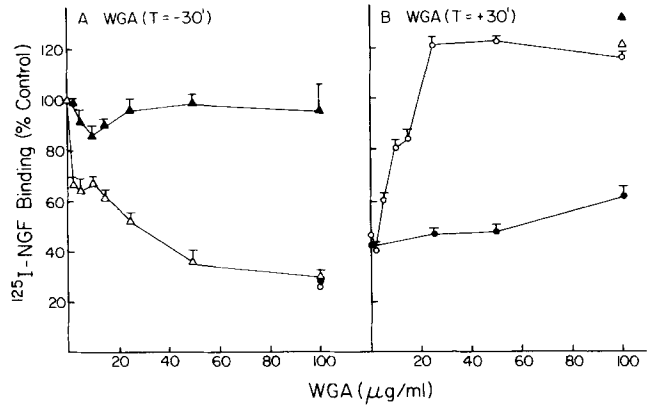

FIGURE 2 Inhibition of the effects of WGA on NGF binding by *N*-acetyl-D-glucosamine. (*A*) PC12 cells were preincubated with WGA at the indicated concentration for 30 min at 37°C in the presence and absence of *N*-acetyl-D-glucosamine (GlcNAc). ^{125}I-NGF (100 pM) was added and total binding was determined from cells in the presence (▲) and absence (△) of GlcNAc. Slowly dissociating binding with 100 μg/ml WGA is also shown inthe presence of GlcNAc (●) and in its absence (○). (*B*) PC12 cells in the presence and absence of GlcNAc were incubated first with ^{125}I-NGF (120 pM) for 30 min at 37°C followed by an additional 30-min incubation with the indicated WGA concentration. Slowly dissociating binding in the presence of GlcNAc (●) and in its absence (○) and total binding at 100 μg/ml WGA in the presence (▲) and absence (△) of the sugar were measured. Values for both experiments are expressed as the percent of a control which received neither GlcNAc nor WGA. Error bars indicate the standard deviations.

Since sensory neurons from chick embryo dorsal root ganglion also display two classes of NGF receptors (4), the effect of WGA on these receptors was also examined. Table III shows that WGA produces similar effects on NGF binding to the dorsal root ganglion cells as were observed with PC12 cells. WGA, added 30 min before ^{125}I-NGF, produced a 50% decrease in total ^{125}I-NGF binding to these ganglion cells. The majority (82%) of this binding was slowly dissociating compared to 48% in the control cells. WGA added after ^{125}I-NGF had less of an effect on total binding but converted 98% of the binding to a slowly dissociable form. The similar effects of WGA and PC12 on both NGF binding and dorsal root ganglion cells indicates that the effect may reveal a common property of all NGF receptors.

Association of NGF Receptors with the Triton X-100 Insoluble Cytoskeleton

Schechter and Bothwell (9) recently reported that a proportion of NGF binding cannot be solubilized with 0.5% Triton X-100 and suggested that this receptor-bound NGF (equivalent to their slow receptor binding) may be associated with the cytoskeletal matrix. Furthermore, it is known that the lectin, Con A, attaches two platelet cell surface glycoproteins to a Triton X-100 insoluble cytoskeleton (14). These findings raise the possibility that WGA might induce an attachment of the NGF receptor to cytoskeletal components. To test this hypothesis, the Triton X-100 solubility of NGF binding was investigated in the presence and absence of WGA. For this purpose, the technique of Schechter and Bothwell (9), of rapidly solubilizing PC12 cells by centrifuging them through a 0.3 M sucrose solution containing 0.5% Triton X-100, was used. Longer solubilization times (30 s to 4 min) produced results similar to the ones described with this rapid procedure (data not shown). ^{125}I-NGF, which pelleted through this Triton X-100 solution, was considered to be Triton X-100 insoluble. Electron microscopy of PC12 cells after Triton X-100 extraction revealed nuclei and intact cytoskeletal networks and the loss of most membrane structure (data not shown). Similar findings have been observed for a number of cell lines after Triton X-100 extraction (12, 15).

In the absence of WGA, the majority of ^{125}I-NGF bound to PC12 cells was solubilized by centrifugation through 0.5% Triton X-100 and was not associated with the Triton X-100 insoluble pellet (Table II). On the other hand, when cells were first incubated with ^{125}I-NGF and subsequently treated with WGA, 90% of the cell-bound NGF became Triton X-100 insoluble. This increase in the Triton X-100 insolubility correlated quantitatively with the increase in slowly dissociable binding after WGA addition. Lectins, which did not affect NGF binding, also did not affect the proportion of binding associated with the Triton X-100 insoluble pellet. Thus, the cross-linking ability common to a variety of lectins is insufficient by itself to cause the association of the NGF receptor complex with Triton X-100 insoluble material.

The effects of WGA on NGF binding occur very rapidly. The time courses of the WGA-induced conversion of the ^{125}I-NGF-receptor complex to slow dissociating receptors and to Triton X-100 insolubility at 37° and 4°C are shown in Fig. 3. ^{125}I-NGF was incubated first for 30 min at either 37° or 4°C with PC12 cells. Within 30 s after subsequent WGA addition

TABLE III

Effect of WGA on ^{125}I-NGF Binding to Chick Embryo Dorsal Root Ganglion Cells

Treatment	Total binding	Slowly dissociating binding	
	pg ^{125}I-NGF/ 10^5 cells	*pg ^{125}I-NGF/ 10^5 cells*	*% Total*
None	86.3 ± 7.4	41.6 ± 2.0	48.2
First WGA, then ^{125}I-NGF	43.8 ± 3.7	35.9 ± 2.7	81.9
First ^{125}I-NGF, then WGA	63.7 ± 11.6	62.3 ± 2.3	97.8

Chick embryo dorsal root ganglion cells were prepared as previously described (12). WGA (50 μg/ml) was added 30 min before or after ^{125}I-NGF (3 nM). Total and slowly dissociating binding were assayed 60 min after ^{125}I-NGF addition. Results represent the mean and standard deviation of triplicate determinations.

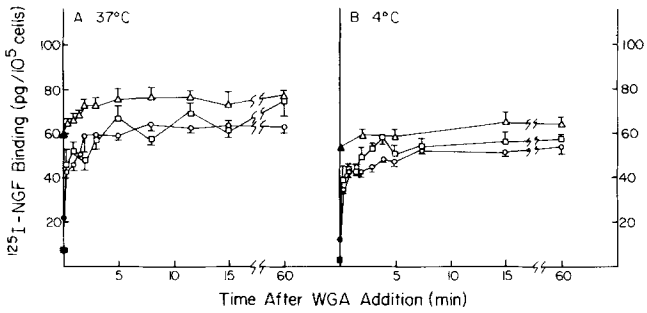

FIGURE 3 Kinetics of the effects of WGA on NGF binding. PC12 cells were incubated with either 150 pM ^{125}I-NGF at 37°C (*A*) or 190 pM ^{125}I-NGF at 4°C (*B*). 30 min after ^{125}I-NGF addition, WGA (50 μg/ml) was added and total (△), slowly dissociating (○), and Triton X-100 insoluble (□) binding were determined as a function of time after WGA addition. The 0-min time-points (▲, ●, ■) represents these binding parameters before WGA addition. Values are the mean of triplicate determinations and bars indicate the standard deviations.

at either temperature, slowly dissociating and Triton X-100 insoluble ^{125}I-NGF increased several fold. Maximum response to WGA occurred after ~5 min. The kinetics of the appearance of the WGA-induced slowly dissociating receptors and Triton X-100 insoluble ^{125}I-NGF were similar, suggesting the two phenomena may be related. In contrast, the appearance of ^{125}I-NGF binding to slowly dissociating receptors in the absence of WGA shows kinetics which reach completion only after 20 min at 37°C (see Fig. 1).

The effects of WGA on NGF binding and cytoskeletal attachment require the continuous presence of the lectin and can be rapidly reversed with its removal. WGA-induced, slowly dissociating receptors were converted back to rapidly dissociating receptors when the cells were centrifuged and resuspended in 100 mM *N*-acetyl-D-glucosamine to dissociate cell-bound WGA (Fig. 4*A*). Within 5 min after sugar addition, the proportion of slowly dissociating binding relative to total decreased from 90% to 45%, while total binding remained the same, suggesting that the WGA-induced slowly dissociating receptors were converted back to their original rapidly dissociating form. The association of the NGF-receptor complex with the Triton X-100 cytoskeleton was also reversible. Fig. 4*B* shows that 80% of the NGF bound to the Triton X-100 cytoskeleton from WGA-treated cells was dissociated within 10 min by adding *N*-acetyl-D-glucosamine. These results indicate that WGA does not permanently alter the binding properties of the NGF receptor or covalently attach it to the cytoskeleton.

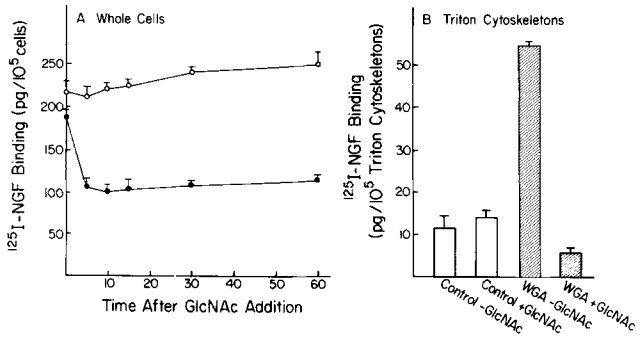

FIGURE 4 Reversibility of WGA-induced slowly dissociating and Triton X-100 insoluble ^{125}I-NGF binding by *N*-acetyl-D-glucosamine. (*A*) ^{125}I-NGF (750 pM) was added to PC12 cells at 37°C for 30 min followed by an additional 30-min treatment with 50 μg/ml WGA. At this time, both total and slowly dissociating binding were determined. Cells were centrifuged subsequently for 5 min at 1,000 *g* and resuspended in binding buffer containing ^{125}I-NGF (750 pM) and 100 mM *N*-acetyl-D-glucosamine. Total (○) and slowly dissociating (●) binding were determined at the indicated times thereafter. (*B*) ^{125}I-NGF (750 pM) was added to cells for 30 min at 37°C followed by an additional 30-min incubation in the presence or absence of WGA (50 μg/ml). Next, four aliquots (400 μl) of WGA untreated and treated cells were centrifuged at 2,000 *g* for 1 min in a Beckman microfuge. Supernatants were removed, and cell pellets were resuspended in 400 μl of cold 0.5% Triton X-100 buffer. Triton X-100 insoluble material was centrifuged at 10,000 *g* for 1 min in a microfuge, the supernatants removed, and the Triton X-100 insoluble pellets resuspended in binding buffer with or without 100 mM *N*-acetyl-D-glucosamine for 10 min at 37°C. ^{125}I-NGF which remained bound to Triton X-100 insoluble material was measured by centrifuging three aliquots (100 μl) over 0.15 M sucrose buffer. Results from four determinations are expressed as pg of ^{125}I-NGF bound to Triton X-100 cytoskeletons derived from 10^5 cells. Bars indicate the standard deviations.

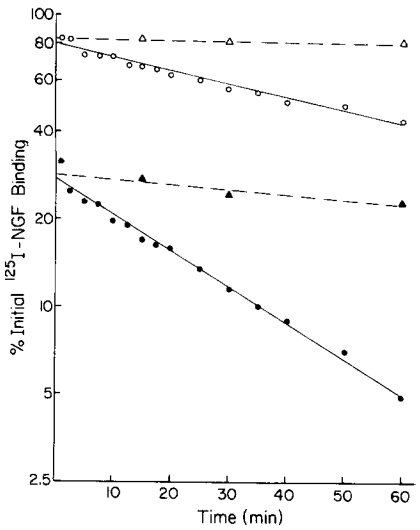

FIGURE 5 Dissociation kinetics of ^{125}I-NGF bound to slowly dissociating receptors on WGA-treated and -untreated cells. PC12 cells were incubated first with 1 nM ^{125}I-NGF at 37°C for 30 min and for an additional 30 min in the presence or absence of WGA (50 μg/ml). Total binding was determined at this time. WGA treated and untreated cells were subsequently centrifuged for 5 min at 500 *g* at 4°C. Supernatant containing ^{125}I-NGF was removed, and cells were resuspended in 7.5 ml of cold binding buffer. After a 15 min incubation at 4°C to displace the majority of ^{125}I-NGF bound to rapidly dissociating receptors, cells were centrifuged as described above, the supernatants removed, and the cells resuspended in their original volume of binding buffer plus or minus WGA (50 μg/ml). Unlabeled NGF (1 μM) was added and dissociation of ^{125}I-NGF from slowly dissociating binding sites was determined at 37°C (●, WGA-untreated; ○, WGA-treated cells) and 4°C (▲, WGA-untreated; △, WGA-treated cells). Triplicate determinations were made at each time-point. Results presented here have been averaged from three experiments and are expressed as the percent of the initial total binding before dissociation (275 pg/10^5 WGA-treated cells; 298 pg/10^5 WGA-treated cells). The line through the data points is the best fit provided by linear regression.

Characteristics of the WGA-induced, Slowly Dissociating Receptor

The dissociation properties of the NGF receptor in the presence of excess unlabeled NGF from WGA-treated and control cells are shown in Fig. 5. Most of the rapidly dissociating binding was first removed by suspending cells in NGF-free medium for 15 min at 4°C. Dissociation from the slowly dissociating receptors, initiated by adding excess unlabeled NGF was then measured at 37° and 4°C. At the lower temperature, the rate of dissociation from control cells was very slow while that from WGA-treated cells was negligible. At 37°C, the rates of dissociation were significantly faster with a $t_{1/2}$ of 25 min for control cells and 65 min for the WGA-treated cells. The rate of dissociation from the WGA-induced slowly dissociating receptor is therefore similar but perhaps not equal to the rate of dissociation from preexisting slowly dissociating receptors. Although the dissociation rate for WGA-treated cells was slower than that from control cells, over twice as much ^{125}I-NGF (measured as pg/10^5 cells) dissociated from the WGA-treated cells compared to control cells, indicating that ^{125}I-NGF was not dissociating exclusively from a population of receptors existing before WGA addition. In both control and WGA-treated cells, most of the ^{125}I-NGF which dissociated in the first 30 min was judged to be intact by trichloracetic acid

precipitability. The actual figures were 80% and 92% intact ^{125}I-NGF for control and WGA-treated cells, respectively. After a period of 1 h, a total of 78% and 89% intact ^{125}I-NGF released into the medium was observed in the respective situations. The dissociation data show that WGA converts the rapidly dissociating receptor into a form similar to the preexisting slowly dissociating receptor. However, the data is not precise enough to determine whether binding in the presence of WGA reflects a homogeneous population of WGA-induced receptors or a mixture of preexisting and WGA-converted slowly dissociating receptors.

The ratio of rapidly and slowly dissociating binding depends upon the concentration of ^{125}I-NGF used in the assay (Fig. 6). In untreated cells, NGF bound primarily to slowly dissociating receptors at concentrations of 50 pM or less, but, as concentrations of ligand were increased, a greater proportion of the NGF bound to rapidly dissociating sites. This result indicates that slowly dissociating receptors have a higher affinity but lower capacity for NGF at equilibrium. When WGA was added after ^{125}I-NGF, >90% of the NGF bound was present in a slowly dissociating form at all concentration of ligand tested. Thus, WGA-induced conversion of receptors appears to be complete over a wide range of concentrations of ^{125}I-NGF bound to the cell surface.

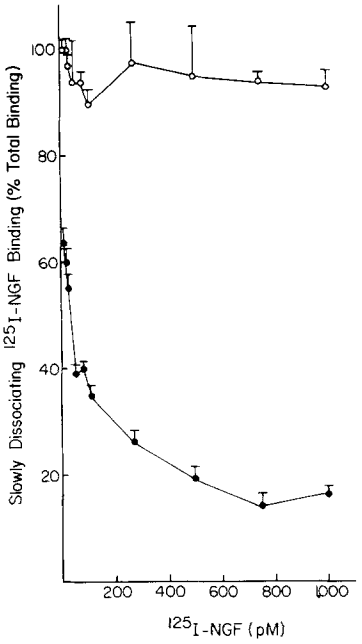

FIGURE 6 Proportion of slowly dissociating binding as a function of ^{125}I-NGF concentration. ^{125}I-NGF was added to PC12 cells at the indicated concentration. After a 30-min incubation at 37°C, WGA (50 μg/ml) was added to one aliquot but not the other. The incubation with ^{125}I-NGF was continued for an additional 30 min and the cells then assayed for total and slowly dissociating binding. Slowly dissociating binding in control cells (●) and WGA-treated cells (○) are expressed as percent of total binding, and bars indicate standard deviations.

TABLE IV

Trypsin Sensitivity of ^{125}I-NGF Binding

Treatment	Total binding	Slowly dissociating binding		Trypsin-resistant binding	
	pg ^{125}I-NGF/ 10^5 cells	*pg ^{125}I-NGF/ 10^5 cells*	*% Total*	*pg ^{125}I-NGF/ 10^5 cells*	*% Total*
None	237.7 ± 5.8	83.2 ± 9.8	35.0	70.9 ± 0.8	29.8
WGA	249.9 ± 4.0	234.2 ± 8.8	93.7	199.1 ± 6.7	79.7

PC12 cells were incubated with ^{125}I-NGF (350 pM) at 37°C. After 30 min, half of the sample received 50 μg/ml WGA. At 60 min, cells were assayed for total, slowly dissociating, and trypsin-resistant binding. Trypsin resistance was determined by incubating 400 μl of cells with 0.5 mg/ml bovine trypsin at 4°C for 30 min. Results represent the mean and standard deviation of triplicate determinations.

Landreth and Shooter (3) demonstrated that NGF bound to slowly dissociating receptors is trypsin-resistant, while NGF bound to rapidly dissociating receptors could be degraded. This resistance to proteolytic degradation could not be explained by the internalization of the receptor. ^{125}I-NGF bound to WGA-induced slowly dissociating sites behaved similarly with regard to trypsin sensitivity (Table IV). The conversion of receptors to a slowly dissociating state induced by WGA was paralleled by an increase in trypsin-resistance of NGF binding from 30% to 80%.

Metabolic energy poisons do not affect the WGA-induced conversion of NGF receptors. Table V shows effects of WGA on NGF binding to cells which were preincubated in glucose-free binding buffer containing 2-deoxy-glucose, sodium azide, and antimycin D. These agents rapidly lower the ATP content of PC12 cells by 80–90% (Herman, E. E., and E. E. Reynolds, Personal communication). In the absence of WGA, energy-depleted cells exhibited a decrease in total binding and a lower proportion of slowly dissociating binding than control cells. However a similar WGA-induced conversion of receptors occurred in both untreated and energy-depleted cells. The WGA-induced association of the NGF-receptor complex with the Triton X-100 cytoskeleton also took place in the presence of energy poisons. These results indicate that the effects of WGA on the NGF receptor occur via an energy independent pathway.

DISCUSSION

The binding of NGF to cell surfaces is most consistent with the presence of two receptor subtypes (4). However, in PC12 cells, the nature and possible interrelation of these two receptor populations is not yet clear. Landreth and Shooter (3) proposed that NGF binds initially to rapidly dissociating receptors, some of which are subsequently converted to slowly dissociating receptors. Schechter and Bothwell (9), on the other hand, report that the two receptor types exist before NGF is added and that no interconversion of receptor occurs. The experiments described here demonstrate that rapidly dissociating receptors can be converted to slowly dissociating receptors by the lectin WGA. They also suggest that the cytoskeleton is implicated in the conversion process.

TABLE V

Effect of Metabolic Energy Poisons on WGA-induced Receptor Conversion and Cytoskeletal Attachment

Treatment	Total binding	Slowly dissociating binding		Triton X-100 insoluble binding	
	pg ^{125}I-NGF/10^5 cells	*pg ^{125}I-NGF/10^5 cells*	*% Total*	*pg ^{125}I-NGF/10^5 cells*	*% Total*
None	251.9 ± 13.0	71.2 ± 2.8	28.3	29.3 ± 9.2	11.6
Energy inhibitors	142.9 ± 3.4	22.3 ± 1.9	15.6	14.3 ± 3.1	10.0
WGA	281.5 ± 7.3	246.1 ± 3.3	87.4	236.4 ± 5.8	84.0
WGA and energy inhibitors	161.6 ± 10.2	124.3 ± 5.4	76.9	132.8 ± 2.6	82.2

PC12 cells were suspended in binding buffer or glucose-free binding buffer containing 10 mM 2-deoxy-D-glucose, 10 mM sodium azide, and 100 nM antimycin D for 15 min at 37°C. ^{125}I-NGF (450 pM) was added and 20 min later WGA (50 μg/ml) was added to half of the samples. At 40 min, cells were assayed for total, slowly dissociating, and Triton X-100 insoluble binding. Results represent the mean and standard deviations of triplicate determinations.

Several lines of evidence indicate that the WGA-induced receptor conversion occurs at the cell surface and is not the result of internalization of rapidly dissociating receptors. The receptor conversion by WGA is observed at 4°C and in the presence of metabolic energy inhibitors, conditions which have been shown to block endocytosis (16, 17). Furthermore, the receptor conversion is essentially complete within seconds, even at 4°C, while endocytosis of hormone-receptor complexes requires minutes at 37°C before it is observed (18). NGF dissociating from WGA-induced, slowly dissociating sites is also mostly intact and has not been degraded by passage through lysosomes. Finally, WGA-induced, slowly dissociating receptors can be converted back to their original rapidly dissociating form by displacing cell-bound WGA with *N*-acetyl-D-glucosamine. This latter experiment argues that a reversible change in the NGF receptor induced by WGA is responsible for the observed effects on NGF binding.

Whether it is added before or after ^{125}I-NGF, WGA converts most receptors to a slowly dissociating form. When WGA is added beforehand, a 50% decrease in binding due to a loss of site numbers is observed. It is unclear whether this decrease in binding is related to the receptor conversion; however, it is conceivable that two or more receptors are required to form a single functional slowly dissociating receptor, as would be the case if a NGF dimer bound simultaneously to two receptors. On the other hand, when WGA is added after ^{125}I-NGF, the ^{125}I-NGF remains bound in the process of receptor conversion and no loss of binding is observed. A decrease in total binding due to a loss of receptors will only manifest itself after binding equilibrium has been reestablished. Since the ^{125}I-NGF bound to newly converted, slowly dissociating receptors dissociates at an extremely slow rate in the absence of unlabeled ligand, binding equilibrium may only be attained after many hours.

In addition to receptor conversion, WGA produces an association of receptor-bound NGF with Triton X-100 insoluble material. Although this cell fraction contains both nuclear and cytoskeletal elements, the results obtained here are consistent with NGF being associated with the cytoskeleton. The presence of NGF in Triton X-100 insoluble material after WGA addition occurs in seconds, before endocytosis of receptors (18) or nuclear translocation (19) has been shown to occur. Also, the rapid solubilization assay makes binding of NGF to nuclear material an unlikely possibility. Nonetheless, further work is required to establish convincingly a direct linkage of the NGF receptor with cytoskeletal components.

Studies, consistent with the results observed with WGA, have demonstrated that lectins can induce the attachment of cell surface proteins to the underlying cytoskeleton (20). Recently, Painter and Ginsberg (14) provided biochemical evidence that Con A can induce the association of two major platelet membrane glycoproteins to the cytoskeleton. Similar to the observations with the NGF receptor, only 10% of these platelet glycoproteins are associated normally with the cytoskeleton, but are 90% associated 15–30 s after addition of Con A.

One explanation of the effects of WGA on NGF binding to PC12 cells is provided by the mobile receptor hypothesis proposed by Boeynaems and Dumont (21) and Jacobs and Cuatrecasas (8). This hypothesis states that hormone receptors interact with effector molecules in the plane of the membrane to alter receptor affinity and activity. Effector-receptor complexes have greater affinity for ligand than the receptor alone. Binding of hormone to two populations of receptors will be observed if a limiting number of effector proteins are available or because of a limiting affinity of the receptor for the effector. This model has been used successfully to explain the curvilinear Scatchard plots and enhanced dissociation kinetics in the presence of unlabeled ligand exhibited by several receptor-hormone systems including NGF (4).

The conversion of a heterogeneous population of NGF receptors to a population of slowly dissociating receptors could be the result of WGA either stabilizing a receptor-effector complex or mimicking the effector itself. The cross-linking ability of WGA may be important in producing such an effector response. Recent studies have shown that anti-insulin antibodies when added with ^{125}I-insulin and liver cell membranes produce an apparent conversion of low to high affinity sites (22). Fab fragments without cross-linking ability do not produce this effect.

One candidate for an effector molecule for the NGF receptor is either the cytoskeleton or a cytoskeleton-associated protein. The data described here suggests that WGA can induce the association of the NGF receptor with the cytoskeleton. The time course of this cytoskeleton association as well as its magnitude is paralleled by the conversion of rapidly to slowly dissociating receptors suggesting that the two events may be related. Recently, Schechter and Bothwell (9) have provided evidence for the association of one type of NGF receptor in PC12 cells with the cytoskeleton using an identical Triton X-100 solubilization assay. Their experiments indicate that NGF receptors with slowly dissociating kinetics are also cytoskeletally attached. This result is similar to those given here in that all of the WGA-induced, slowly dissociating receptors are also associated with the cytoskeleton. Are WGA-induced, slowly dissociating receptors therefore the same as the slowly dissociating receptors normally found on PC12 cells? The rate at which ^{125}I-NGF binds to these two receptors is the same, as also is their trypsin-resistance, arguing in favor of this idea. On the other hand, the somewhat different dissociation kinetics for the two receptors suggest that the two species may not be entirely identical.

The correlation of cytoskeletal attachment of the NGF receptor with the formation of slowly dissociating binding sites is of interest since such events may be important for the internalization and biological action of NGF. Within minutes, NGF has been shown to undergo clustering and subsequent internalization in PC12 cells (23) as well as to produce prominent cell surface ruffling (24), events which may involve the cytoskeleton. The extension of neurites is a demonstration of the long term effects that NGF has on cytoskeletal architecture. These profound cellular changes produced by NGF could involve the attachment of the receptor or a receptor-associated protein to the cytoskeleton. WGA may promote or stabilize the association of the NGF receptor with the cytoskeleton, thus allowing the isolation and characterization of such a complex.

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