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A Comparison of Binding Properties and Structure of NGF Receptor on PC12 Pheochromocytoma and A875 Melanoma Cells

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Rat PC12 pheochromocytoma and human A875 melanoma cells express nerve growth factor (NGF) receptors on their surfaces. Covalent crosslinking of bound ^{125}I-NGF to PC12 or A875 intact cells or plasma membrane-enriched fractions resulted in labelling of a peptide doublet at Mr = 110,000 and a single labelled peptide at Mr = 200,000 for each of the cell and membrane preparations. However, a difference between equilibrium binding properties of NGF-receptor on PC12 and A875 cells was observed. PC12 cells exhibited biphasic binding properties with two apparent binding sites: K_D = 5.2 nM sites and K_D = 0.3 nM sites. The high-affinity PC12 binding sites were trypsin resistant, and ^{125}I-NGF dissociated slowly from them. A875 cells exhibited sites with homogeneous properties (K_D = 1.0 nM), all binding sites were trypsin sensitive, and ^{125}I-NGF dissociated rapidly in the presence of unlabelled NGF. Membrane-enriched fractions from either cell type contained binding sites with a uniform low affinity (K_D = 3 nM) that were trypsin sensitive, and ^{125}I-NGF rapidly dissociated from them. Sixty to 80 percent of binding sites in membranes could be converted to the high-affinity, trypsin-resistant state by addition of wheat germ agglutinin (WGA). The loss of high-affinity, trypsin-resistant sites from PC12 cells during preparation of plasma membrane fractions does not appear to be the result of selective isolation of low-affinity sites or proteolytic degradation since there is a loss of ^{125}I-NGF binding immediately after cell lysis which is not blocked by protease inhibitors. Also, high-affinity, trypsin-resistant binding sites are not found associated with other cell fractions. The differences between receptor properties on PC12 cells and on A875 cells apparently are the result of differences in the respective intracellular environments. Thus, significant structural homology exists between receptors on A875 and PC12 cells. Cell components other than the binding unit of the NGF receptor may be responsible for the different properties of receptor.

Key words: NGF receptor, NGF binding, PC12 cells, nerve growth factor

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Nerve growth factor (NGF) is required in the development and maintenance of the sympathetic nervous system and specific sensory neurons. NGF causes responsive neurons to increase in size and to extend axonlike processes and specifically induces enzymes characteristic for adrenergic neurons and adrenal medullary cells [for review see 1]. Specific binding of NGF to cell-surface receptors has been demonstrated for sensory and sympathetic neurons [2,3], the clonally derived PC12 rat pheochromocytoma [4], and the A875 human melanoma [5]. The PC12 and A875 cell lines can be grown under conventional culture conditions in the absence of NGF, offering the advantage of not requiring exposure to NGF prior to experimental manipulation. Additionally, the A875 cells grow to a high density in culture and have $5–10 \times 10^5$ NGF receptors per cell. Thus, the A875 cell line provides a valuable source for purifying the NGF receptor.

NGF receptor on PC12 cells mediates the transformation of the pheochromocytoma to a neuronlike state in the presence of NGF. Although no similar neuronlike transition occurs in A875 cells grown in the presence of NGF, these cells respond to NGF with increased cell survival in serum-depleted medium* [5]. Two forms of NGF receptor have been described on neurons and PC12 cells. These two forms of receptor have distinguishable kinetic and biochemical properties. A low-affinity form of NGF receptor is identified experimentally by the rapid dissociation of prebound ^{125}I-NGF in the presence of a high concentration of unlabelled NGF and by its susceptibility to trypsin [6]. A high-affinity form of receptor has also been described from which prebound ^{125}I-NGF dissociates slowly and which is trypsin insensitive [6,7] and insoluble in low concentrations of Triton X-100 [7,8]. This slowly-dissociating, trypsin-resistant form of NGF receptor may be associated with the cytoskeleton [7,8]. Alternatively it may be associated with the nucleus [9] or involved in a receptor-mediated internalization process for NGF [10]. Recent evidence strongly indicates that the low-affinity form of receptor can be converted to the high-affinity form [6,11]. This conversion can be induced experimentally by treatment of cells, membranes, detergent-solubilized extracts or reconstituted receptor with wheat germ agglutinin (WGA).

We report here that the receptor from the A875 and PC12 cell lines have very similar properties. Although the quantity of receptor differs on the PC12 and A875 cells and plasma membranes prepared from them, the Mr of the predominant peptides covalently cross-linked to specifically bound ^{125}I-NGF are indistinguishable in the two cell types. Both high- and low-affinity forms of receptor were observed on PC12 cells, whereas only a homogenous population of receptor with a single affinity was detected on A875 cells. Enriched plasma membrane fractions prepared from both types of cells exhibited a single homogenous form of receptor with similar kinetic and biochemical properties. The evidence presented supports the hypothesis that the NGF receptor is structurally very similar in both types of cells. The receptor is convertible between two affinity states, and the differences observed between receptor on PC12 and A875 cells is best explained by cell components interacting with the receptor in intact PC12 cells but not A875 cells. The interaction of these cell components with the PC12 NGF receptor is lost after lysis and membrane preparation, and the components are functionally uncoupled or absent in A875 cells.

*(L. Watson and G.L. Johnson, unpublished observations)

MATERIALS AND METHODS

Preparation of NGF and ^{125}I-NGF

Purification of NGF from submaxillary glands of adult male mice was as described by Mobley et al [12]. Radioiodination of NGF was performed with immobilized glucose oxidase and lactoperoxidase as has been described in detail previously [11].

Analysis of ^{125}I-NGF binding

Binding of ^{125}I-NGF to cells, cell homogenates, or membranes was determined by a centrifugation assay. Briefly, cells, homogenates, or membranes suspended in Hepes buffered (pH 7.4) Krebs-Ringer saline containing 0.1% albumin (KRH/A) were incubated with ^{125}I-NGF at concentrations and temperatures as shown. Three replicate 100 μl samples were centrifuged in separate 0.5 ml polypropylene centrifuge tubes in a Beckman microfuge (10,000g) for 1 min. The supernatant was aspirated and the bottom of each tube containing the cell or membrane pellet was cut off and counted in a gamma counter. Nonspecific binding was determined by parallel incubations in the presence of 10 μg/ml unlabelled NGF. Typically, nonspecific binding was 10% or less of total binding, and the standard deviation for three replicates was within 10% of the mean.

Analysis of the slowly dissociating and trypsin-resistant form of the receptor was based on techniques originally described by Yankner and Shooter [9], Landreth and Shooter [6], and Schechter and Bothwell [7]. After equilibration of ^{125}I-NGF binding to cells or membranes, 10 μl of 100 μg/ml NGF or 10 μl of 500 μg/ml trypsin was added to triplicate 100 μl cell- or membrane-containing aliquots. Incubation was continued for 30 min on ice before centrifugation (microfuge) and aspiration of the supernatant. Specific slowly dissociating or trypsin-resistant binding was determined by subtraction of nonspecifically bound ^{125}I-NGF determined as described above.

A875 cells used in binding assays were removed from plastic tissue culture dishes by incubation for 15 min at 37°C with Puck's saline plus 0.5 mM edetic acid (EDTA). Cells were vigorously pipetted to obtain a single cell suspension and washed and resuspended in KRH/A before use in binding assays. PC12 cells, which do not firmly adhere to plastic tissue culture surfaces, were suspended by vigorous pipetting and washed and resuspended in KRH/A before use. Greater than 90% viability could be maintained for several hours with these harvesting procedures.

Preparation of membranes

The human melanoma cell line, A875, was a gift from Dr. George Todaro. The cells were grown in 850-cc roller bottles in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. PC12 cells were obtained from Dr. John Wagner (Sidney Farber Cancer Research Institute) and grown on 150-mm plastic tissue culture dishes in DMEM supplemented with 10% fetal bovine serum and 10% heat-inactivated horse serum.

A875 cells were removed from roller bottles by scraping with a rubber policeman. PC12 cells were removed by vigorous pipetting of harvesting solution onto the surface of the 150-mm dishes. Harvesting solutions and membrane isolation solutions contained a protease inhibitor cocktail containing 1 mM phenylmethylsulfonyl fluo-

ride, 20 Kallikrein inhibitory units (KIU) ml aprotinin, and 100 μg/ml bacitracin. Either of two methods was used to lyse A875 and PC12 cells for preparation of plasma membrane-enriched fractions. The resulting membrane fractions were not significantly different. In the first method membranes were obtained from washed cells by a modification of the method of Thom et al [13]. Cells were swollen in 20 mM borate, 0.2 mM EDTA pH 10.1 for 10 min. The suspension was homogenized in a Polytron P10 at a setting of 3.5 for 10 sec. After addition of eight volumes of 0.5 M borate pH 10.2, the homogenate was centrifuged at 500g, and the resulting supernatant was centrifuged at 20,000g for 30 min. The pellet was resuspended in phosphate-buffered saline (PBS) and layered over a sucrose step gradient (20% and 40% sucrose w/w, in PBS). After centrifugation at 100,000g for 60 min the plasma membrane-enriched fraction was collected at the 20%-40% interface. Membranes were removed, diluted in PBS, and centrifuged at 30,000g for 20 min. The resuspended pellet was stored at −85°C. Approximately 50 mg of membrane protein was obtained from 50 roller bottles.

Nitrogen cavitation was used as an alternative method of cell lysis. In this technique either A875 or PC12 cells were suspended in a lysis buffer consisting of 20 mM NaH_2PO_4, 2 mM MgCl, and 1 mM EDTA at pH 7.4. The cell suspension was placed on ice in a nitrogen bomb, and gaseous N_2 was used to raise pressure to 500 psi. Cells were allowed to equilibrate for 20 min before being expelled from the chamber, thus undergoing a rapid decrease in pressure and cell lysis. After lysis, cell homogenates were processed for membrane-enriched fractions as described above.

Crosslinking of ^{125}I-NGF to PC12 and A875 cells or membranes

The crosslinking of ^{125}I-NGF to binding peptides was essentially identical to that described by Johnson et al [14] for ^{125}I-glucagon crosslinking to rat liver membranes. ^{125}I-NGF was bound to the membranes or cells as described above. The membranes or cells were washed in ice-cold phosphate-buffered saline, and hydroxysuccinimidyl-azidobenzoate (HSAB) was added to give a 50 μM final concentration. The mixture was incubated in the dark for 2–5 min and photolyzed for 8 min using a 40-W mercury lamp 15 cm from the mixture in a quartz cuvette maintained at 4°C. The reaction was stopped by removing the uv source and adding TRIS-HCl, pH 8.0, to a concentration of 20 mM. The membranes or cells were diluted, centrifuged, resuspended, and solubilized in 1% sodium dodecylsulfate (SDS) and reduced with 5% 2-β-mercaptoethanol and boiled for 5 min. One-dimensional SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli [15] as described by O'Farrell [16] using 7.5% acrylamide concentrations in the separating gel. Gels were stained and fixed in 2% Coomassie blue, 50% acetic acid, and 10% ethanol, then destained in 10% acetic acid and 10% ethanol. Dried gels were used to expose Kodak X-Omat R film with Dupont lightening-plus intensifying screens at −85°C.

Internalization of ^{125}I-NGF

Single cell suspensions of A875 or PC12 cells were obtained as described above for binding assays. The cells were suspended in DMEM containing 10 mg/ml bovine serum albumin (BSA) at a concentration of 5×10^6 A875 cells/ml or 1×10^7 PC12 cells/ml. The cells were incubated with 1 nM (final concentration) ^{125}I-NGF for 60 min at 0°C. Aliquots also containing 10 μg/ml unlabelled NGF were incubated in parallel to determine non-specific binding and uptake. Samples were washed with cold PBS (pH 7.4) and resuspended to the original volume in DMEM with 10 mg/ml

BSA. The samples were warmed on a waterbath shaker at 37°C and 350-μl samples were taken at each time point as indicated. The sample taken was cooled for 5 min on ice and microfuged 1 min to separate the cells. Three 100-μl samples of the supernatant were taken for counting and trichloracetic acid (TCA) precipitation. The cell pellet was resuspended in 350 μl DMEM + 10 mg/ml BSA + 0.1% trypsin. Three 100-μl samples of the pellet suspension were carefullly layered over 200 μl 0.3 M sucrose. After incubation for 15 min on ice, the cells were microfuged 1 min through the sucrose, then quick frozen in dry ice/95% ETOH. Tips of the microfuge tubes were cut off, and both cells and media were counted separately for ^{125}I.

Media reserved for TCA precipitation before addition of trypsin as described above was incubated 30 min on ice with 10% TCA, microfuged, and the supernatant removed and saved. The pellet was washed with 5% TCA, and the wash supernatant was pooled with the original TCA supernatant. Pellets and combined supernatants were counted separately for ^{125}I.

RESULTS

The heterobifunctional crosslinking reagent hydroxysuccinimydyl-p-azidobenzoate (HSAB) was used to covalently label peptides to which ^{125}I-NGF specifically bound on cells and enriched plasma membrane fractions from PC12 and A875 cells. As shown in Figure 1, peptides of very similar Mr are labelled on PC12 cells and membranes and on A875 cells and membranes. The amount of labelling was greater with A875 cells and membranes than with PC12 cells and membranes, which corresponds to the relative abundance of receptor reported for each type of cell [4,5]. Two bands whose appearance was inhibited by unlabelled NGF were most prominent. These bands corresponded to a doublet of peptides with average Mr = 110,000 and a single band at Mr = 200,000. Several other bands were also observed at intermediate Mr's, but their labelling intensity was variable and these were less prominent than the major bands at Mr = 110,000 and 200,000. These results suggest that there is significant homology between NGF receptor on PC12 and A875 cells. Also, the similar Mr's on cells and membranes indicate that significant proteolysis of receptor does not take place during preparation of membrane fractions.

A difference in ^{125}I-NGF equilibrium binding properties was observed for A875 and PC12 cells (Fig. 2A,B). Scatchard analysis revealed that binding of ^{125}I-NGF to PC12 cells was biphasic and contained two apparent binding sites with Kd_1 = 5.2 nM and Kd_2 = 0.3 nM. Scatchard analysis of ^{125}I-NGF binding at equilibrium to A875 cells, however, revealed only one apparent binding site with Kd = 1.0 nM. Additionally, analysis of the amount of ^{125}I-NGF not dissociated at 0°C in the presence of 10 μg/ml unlabelled NGF—i.e., slowly dissociating NGF—indicated that 20% of the NGF receptor on PC12 cells was slowly dissociable, but none of the NGF receptor on A875 cells was in the slowly dissociating state.

The plasma membrane-enriched fractions from both A875 and PC12 cells contained predominantly one binding site with Kd = 3.4 nM and Kd = 3.9 nM for PC12 and A875 membranes, respectively (Table I; Fig. 3A,B). Of seven PC12 membrane preparations tested, two demonstrated the presence of a high-affinity binding site (K_D < 1 nM), as shown in Figure 3A. In both of these less than 8% of total receptor was high affinity or slowly dissociating. Thus, preparation of plasma membrane-enriched fractions from PC12 cells resulted in a significant decrease or

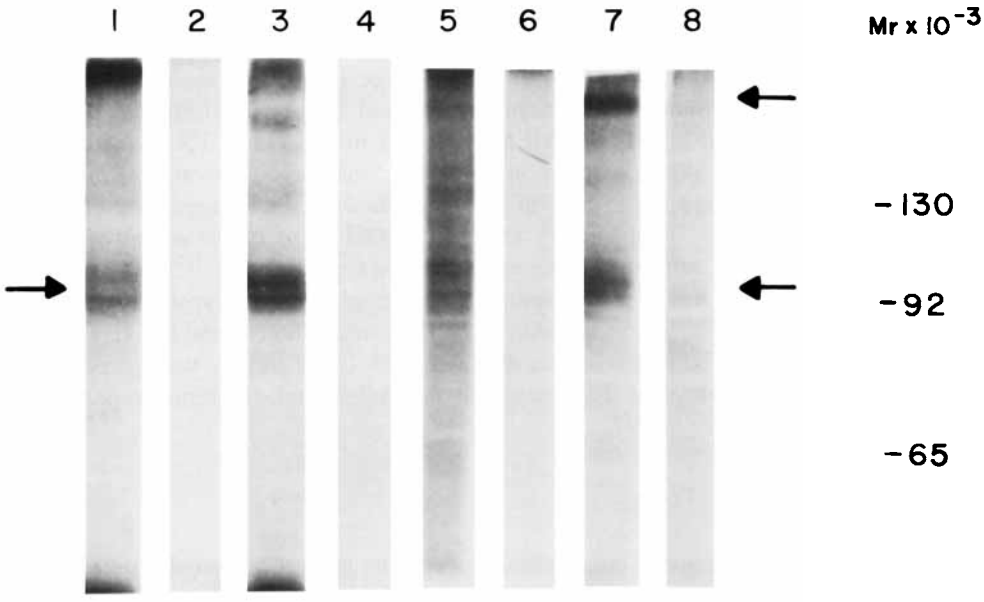

Fig. 1. Autoradiograph of polyacrylamide gel showing covalent crosslinking of ^{125}I-NGF to PC12 and A875 cells and membranes. ^{125}I-NGF was bound and covalently crosslinked to PC12 cells (lanes 1 and 2), A875 cells (lanes 3 and 4), and the plasma membrane-enriched fractions from PC12 (lanes 5 and 6) or A875 cells (lanes 7 and 8) as described in Materials and Methods. Control incubations contained 10 μg/ml unlabelled NGF (lanes 2, 4, 6, and 8). Comigration of standard Mr proteins is indicated at the right. The major crosslinked peptides (indicated by arrows) were a doublet at Mr = 110,000 and a singlet band at Mr = 200,000. The ratio of specific cpm's in the A875 bands to specific cpm's in the PC12 bands was 4:1, which is indicative of the greater amount of NGF receptor in A875 cells and membranes. Time of exposure to x-ray film for each pair of lanes was adjusted to optimize resolution in the preparation of this figure.

complete removal of high-affinity, slowly dissociating binding sites. Analysis of NGF binding to A875 plasma membrane-enriched fractions yielded uniformly linear Scatchard plots with an affinity statistically not significantly different than for A875 cells. The equilibrium binding data for PC12 and A875 cells and membranes are summarized in Table I.

The effect of WGA on NGF binding to receptor was analyzed in both PC12 and A875 intact cells and plasma membrane-enriched fractions (Table II). A small decrease in total specific binding of ^{125}I-NGF was observed for both PC12 and A875 intact cells in the presence of WGA. For the experiment shown (Table II), increased receptor conversion mediated by WGA in PC12 cells was difficult to distinguish since the amount of slowly dissociating, trypsin-resistant binding was relatively high in this sample of untreated cells. However, the effect of WGA on A875 cells was more profound. The amount of slowly dissociating, trypsin-resistant receptor on A875 cells in the absence of WGA was not distinguishable from zero. Addition of WGA led to a slight decline in total specific binding, but the slowly dissociating, trypsin-resistant binding increased at approximately 80% of the total specifically bound ^{125}I-NGF. Thus, NGF receptor on A875 cells can be converted to a slowly dissociating, trypsin-resistant state similar or identical to receptor on PC12 cells.

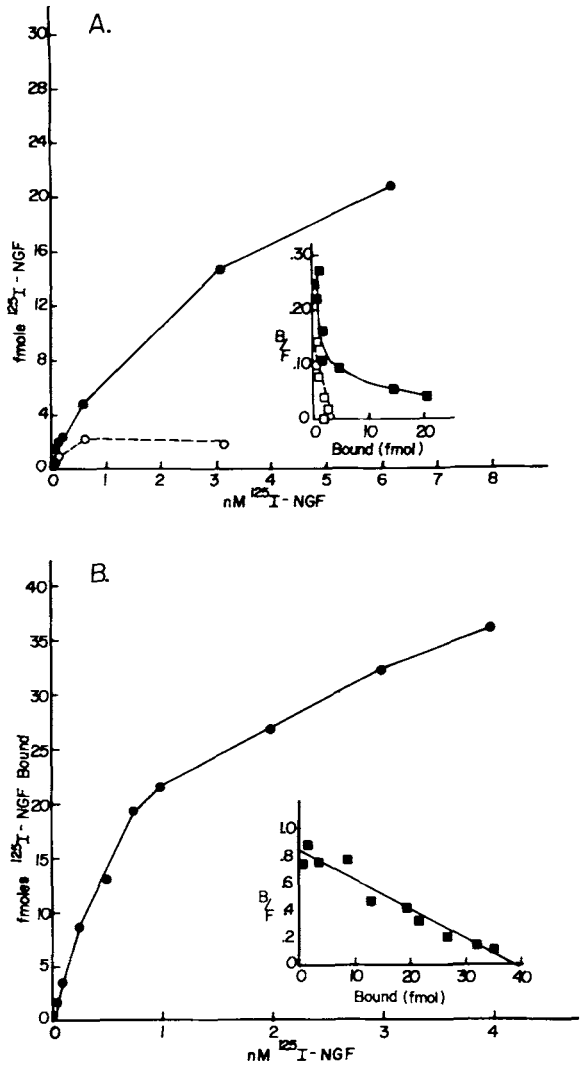

Fig. 2. Binding of ^{125}I-NGF to PC12 cells (A) and A875 cells (B). ^{125}I-NGF at the concentrations indicated was incubated with 5 × 10^6 PC12 cells/ml (A) or 2.5 × 10^5 A875 cells/ml (B). One hundred-microliter aliquots were centrifuged (10,000g) for 1 min, supernatants were removed by aspiration, and ^{125}I-NGF in the cell pellet was counted. The amount of slowly dissociating binding was also determined for PC12 cells (A, □). Scatchard plots for PC12 cells (A, inset) and A875 cells (B, inset) are also shown.

In contrast to the decline in total specific binding of ^{125}I-NGF to intact cells, binding to plasma membrane-enriched fractions of both PC12 and A875 membranes was increased approximately twofold in the presence of WGA. Likewise, trypsin-resistant, slowly dissociating binding was increased to 60%–80% of total specific binding. Although some of the ^{125}I-NGF bound to membranes in the absence of WGA appeared to be slowly dissociating, none of it was concurrently trypsin resistant. Since no high-affinity binding was observed on Scatchard analysis of these membrane

TABLE I. Receptor Equilibrium Binding Properties*

		Low affinity		High affinity	
Cell type	N	K_D(nM)	Capacity	K_D(nM)	Capacity
PC12					
Cells	2	5.2	5.7×10^4 molecules/cell	0.3	1.5×10^4 molecules/cell
Membranes	7	3.4	1.5 pmoles/mg		None
A875					
Cells	6	1.0	8.1×10^5 molecules/cell		None
Membranes	6	3.9	7.2 pmoles/mg		None

*Summary of data obtained by Scatchard analysis for PC12 and A875 cells and plasma membrane-enriched fractions. The values shown are mean values from N experiments. No significant differences were observed between experiments performed at 0°C and room temperature (22°C). Therefore, the results shown were pooled from experiments equilibrated at either temperature.

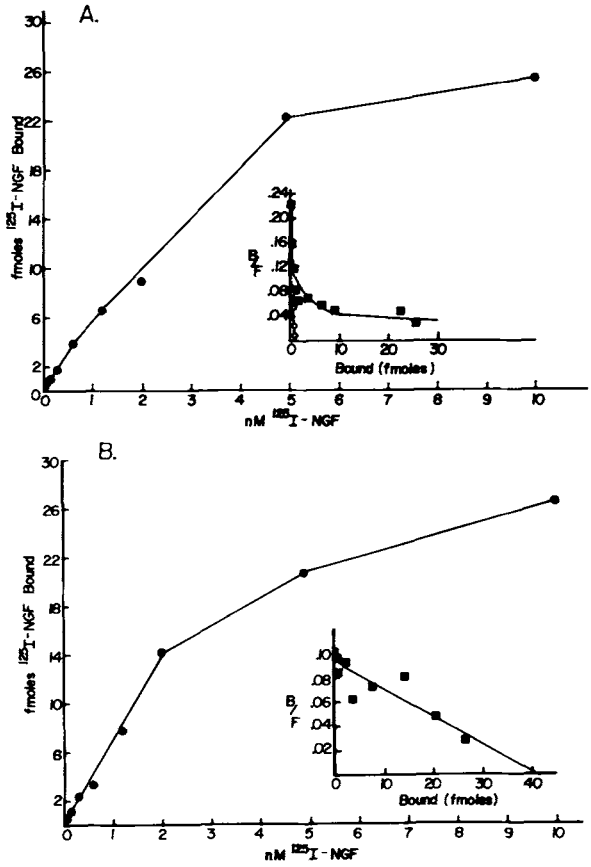

Fig. 3. Binding of ^{125}I-NGF to PC12 (A) and A875 (B) plasma membrane-enriched fractions. One hundred-microliter aliquots of PC12 membranes (500 μg/ml) or A875 membranes (300 μg/ml) were incubated with the concentration of ^{125}I-NGF indicated. Control incubations also contained 10 μg/ml unlabelled NGF. Specific binding was determined as described in Materials and Methods. Scatchard plots for PC12 (A, inset) and A875 (B, inset) membranes are also shown. Slowly dissociating binding was determined in the PC12 membranes (A,○). (●) F moles ^{125}I-NGF bound; (■) Scatchard plots of total specific ^{125}I-NGF bound; (○) Scatchard plot of high affinity ^{125}I-NGF bound.

TABLE II. Effect of Wheat Germ Agglutinin (WGA) on ^{125}I-NGF Binding to PC12 and A875 Cells and Membranes*

	fmoles ^{125}I-NGF bound			
	PC12		A875	
	w/o WGA	+WGA	w/o WGA	+WGA
Cells (10^5)				
Total bound	1.6 ± 0.4	1.1 ± 0.1	26.9 ± 1.1	23.7 ± 2.7
Slow dissociating	1.3 ± 0.3	1.1 ± 0.3	1.5 ± 0.3	18.5 ± 2.1
Trypsin resistant	1.4 ± 0.8	0.9 ± 0.2	2.0 ± 0.9	21.0 ± 1.2
Membrane (50 μg)				
Total bound	7.2 ± 1.0	16.7 ± 2.2	17.1 ± 1.3	39.6 ± 1.8
Slow dissociating	2.3 ± 0.5	11.1 ± 1.9	2.3 ± 0.8	34.6 ± 2.8
Trypsin resistant	0	10.9 ± 1.6	0	23.0 ± 4.3

*Three sets of triplicate 500 μl plastic microfuge tubes containing 100-μl aliquots of cells or membranes were incubated at room temperature for 60 min with 2 nM ^{125}I-NGF. A fourth set of tubes contained 10 μg/ml NGF for determination of nonspecific binding. At the conclusion of the initial incubation period 10 μl PBS was added to one set of tubes, 10 μl trypsin (50 μg/ml final concentration) was added to a second set, and NGF (10 μg/ml final concentration) was added to the third set. Incubation was continued for 30 min on ice; then tubes were microfuged (10000g) for 1 min and supernatants were removed by aspiration. The tip of each tube was cut into a clean test tube and ^{125}I was counted.

fractions, it appears likely that the low level of slowly dissociating binding observed here was artifactual. This also demonstrated the difficulty of analyzing high-affinity binding solely by measurement of slowly dissociating ^{125}I-NGF. The difference observed between the effects of WGA on receptor in intact cells and membrane fractions, however, suggests that receptor has different properties in intact cells than in plasma membrane-enriched fractions although no gross structural changes were evident (Fig. 1).

To determine the cause of the changes in PC12 NGF receptor properties we analyzed the binding properties of receptor at several stages during membrane preparation. Within 5 min after cell lysis an apparent loss in binding activity was observed in cell homogenates (Fig. 4). This loss was not prevented by addition of protease inhibitors. A similar apparent loss in binding was observed in A875 cells, although of lesser magnitude (not shown). The 100,000g supernatant from PC12 cell homogenates indicated the presence of a large amount of NGF binding (Table III). However, ^{125}I-NGF dissociated rapidly from this material, and none was trypsin resistant. Even WGA did not induce trypsin-resistant binding in this supernatant, indicating that the ^{125}I-NGF binding was not to the relevant NGF receptor. A smaller amount of receptor was detected in the dense pellet obtained from sucrose gradients (Table III). While some of this material was slowly dissociating, none was trypsin resistant. However, the receptor present in this fraction was converted to a slowly dissociating, trypsin-resistant state by treatment with WGA. This fraction contained nuclei and other large organelles and possibly cytoskeletal elements with which NGF receptor may be associated [7–9]. However, cytoskeletally associated NGF receptor is reported to be trypsin resistant [7,8], but receptor in this fraction was not trypsin resistant except in the presence of WGA. Additionally, the quantity of slowly dissociating receptor in the sucrose gradient pellet fraction does not account for all of the slowly dissociating

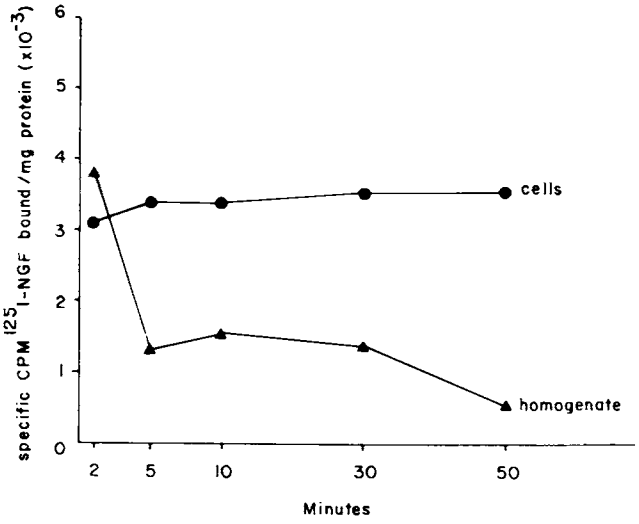

Fig. 4. Time course for the decrease in ^{125}I-NGF binding in PC12 cell homogenates. Ten-milliliter washed cells (6×10^7 cells/ml) were split into two aliquots. One aliquot was lysed by N_2 cavitation at 0°C while the other aliquot was held on ice. Equal volumes of cells or homogenates were quickly added to pairs of test tubes containing ^{125}I-NGF (0.2 nM final concentration with 10 μg/ml unlabelled NGF to one tube of each pair as nonspecific binding control) and HEPES buffered saline with 1 mg/ml albumin at 37°C. Timing was begun upon addition of cells or homogenate to the ^{125}I-NGF containing solution and incubation was continued at 37°C. Three 100-μl aliquots were removed at the times indicated and immediately centrifuged in a microfuge (Beckman Instrument Co.) at 10,000g for 1 min. Nonspecific binding was subtracted from values shown, which are means of three aliquots. The standard error was within 10% of the mean for each value shown. The protein content of cell and homogenate was determined and values were adjusted to cpm specific binding per mg of protein present.

form of the receptor lost after cell lysis. While less than 2% of receptor associated with the plasma membrane fraction was slowly dissociable, 60%–80% of it was converted by treatment with WGA, which indicates that receptor in the membrane fraction retains the ability to be converted under appropriate conditions. These results indicate that the ability to convert receptor to the slowly dissociating, high-affinity state is not lost during cell membrane preparation. Since there is an apparent rapid loss in binding immediately after cell lysis (Fig. 4), the data appear to indicate that the receptor changes rapidly after cell lysis to a lower affinity state.

The experiments using intact cells described above do not clearly distinguish between the trypsin-resistant state of NGF receptor and receptor which has been internalized either via receptor-mediated endocytosis or via nonspecific pinocytosis. Since the slowly dissociating NGF receptor state has been proposed as preliminary to internalization [10] or as potentially necessary for NGF activation [2], the following experiments were performed to distinguish between internalization and the high-affinity state of receptor. The transient increase in trypsin-resistant ^{125}I-NGF at 60–180 min after shifting PC12 cells from 0° to 37°C was accompanied by an increase in TCA-soluble ^{125}I (Fig. 5A). This strongly suggests that NGF is internalized and degraded by PC12 cells. No significant change in trypsin-resistant ^{125}I-NGF nor increase in TCA-soluble ^{125}I was observed over a similar 5-hr incubation period for A875 cells. Thus, PC12 cells internalize and degrade NGF while A875 cells do not.

TABLE III. PC12 Cell Fractions*

Fraction	Protein (mg)	^{125}I-NGF Bound (fmoles/mg protein)	% Slowly dissociating	% Trypsin resistant	Total ^{125}I-NGF bound (pmoles)	+WGA (100 μg/ml) % Slowly dissociating	+WGA (100 μg/ml) % Trypsin resistant
Intact cells	460[a]	78	23%	30%	36	39%	40%
Supernatant[b] (100,000g)	207	84	0	0	17	99%	0
Sucrose gradient pellet	84	62	29%	0	5	54%	73%
Enriched plasma membranes	18	790	<2%	0	14	61%	82%

*Cells were lysed by N_2 cavitation as described in Materials and Methods. The cell lysate was centrifuged at 20,000g. A sample from this supernatant was further centrifuged at 100,000g for 30 min to remove small membrane vesicles and assayed for soluble receptor. The homogenate pellet was loaded onto a 20%–40% sucrose step gradient and centrifuged at 100,000g for 60 min. Enriched plasma membranes were removed from the 20%–40% sucrose interface, and the material that settled through the 40% sucrose was designated sucrose gradient pellet.

[a]Approximately 25% of cells added to N_2 bomb for lysis were not expelled and therefore were not recovered. Thus, less than 100% of cell protein appears in cell fractions.

[b]Binding assayed by polyethylene glycol precipitation of ^{125}I-NGF receptor complex [11].

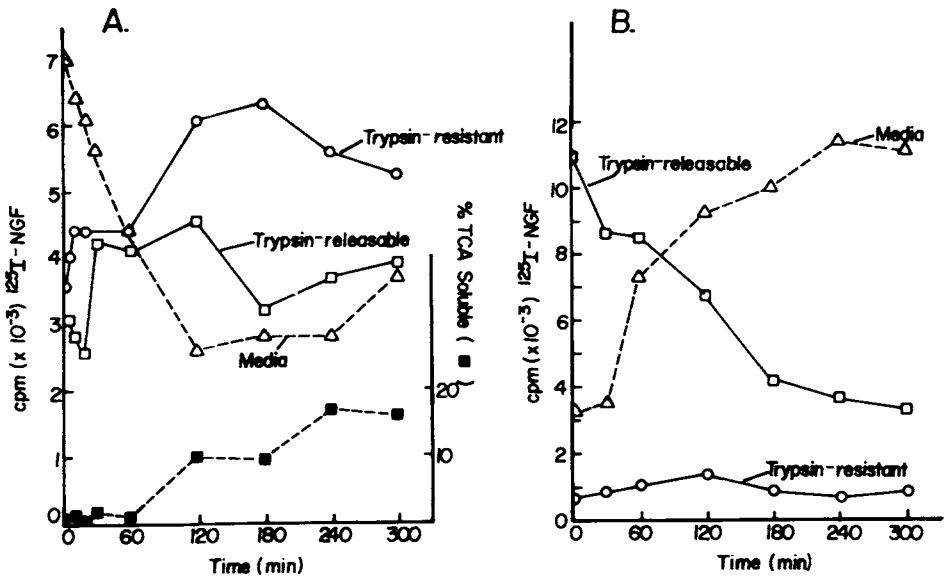

Fig. 5. Temperature-dependent internalization of ^{125}I-NGF following binding at 0°C. ^{125}I-NGF was prebound for 60 min at 0°C to 10^7 PC12 cells/ml (A) or 5×10^6 A875 cells/ml (B). Cells were washed rapidly in the cold, warmed to 37°C for the times indicated, and recooled to 0°C for 5 min before centrifugation. Supernatants were analyzed for total cpm (△) and for cpm soluble in 10% trichloroacetic acid (■). The cell pellet was resuspended in chilled medium containing 0.1% trypsin, layered over 0.3 M sucrose, and incubated on ice for 15 min. Cells were then centrifuged through the sucrose and rapidly frozen in a dry ice-ethanol bath. The cpm in the cell pellet represents bound or internalized trypsin-resistant ^{125}I-NGF (○), and the cpm in supernatants represents surface bound, trypsin-sensitive ^{125}I-NGF (□). The percent TCA-soluble cpm did not exceed 3% at any of the times shown for A875 cells.

Although the amount of trypsin-resistant ^{125}I-NGF increased after transfer from 0° to 37°C, consistent with internalization of NGF, a significant amount (approximately 50%) of trypsin-resistant NGF was evident after incubation at 0°C only (zero time on Fig. 5A). Thus, trypsin-resistant ^{125}I-NGF was present before internalization took place. Therefore, the data are not consistent with the hypothesis that the apparent high-affinity, trypsin-resistant state is due merely to internalization. In addition, while cells do not appear to internalize a significant amount of NGF via receptor-mediated endocytosis, NGF enhances survival of confluent A875 cells in serum-depleted medium [5]. These data suggest that NGF internalization may not be a prerequisite for NGF action.

DISCUSSION

The heterobifunctional photoaffinity reagent HSAB was used to covalently crosslink ^{125}I-NGF to putative receptor molecules. The resulting labelled membrane components on PC12 cells and plasma membrane-enriched fractions were indistinguishable from membrane components labelled on A875 cells and plasma membrane-enriched fractions. The Mr's calculated from autoradiographs of SDS-polyacrylamide gels indicate that receptor peptides on both types of cells consist of a peptide doublet with Mr = 110,000 and a single Mr = 200,000 peptide. A very similar migration pattern for covalently crosslinked ^{125}I-NGF has also recently been observed for highly purified NGF receptor [17]. Significant homology between NGF receptor on A875

and PC12 cells is further demonstrated by characterization of ^{125}I-NGF binding to PC12 and A875 cells and plasma membrane-enriched fractions. Although PC12 cells contain two apparent binding sites with distinct Kd's and A875 cells contain receptor with homogenous affinity, membrane-enriched fractions from both types of cells possess a single form of receptor with a common affinity. This does not appear to be the result of selective isolation of low-affinity binding sites from PC12 cells, since high-affinity binding sites were not detected in any PC12 cell fraction after cell lysis. In fact, a loss of total binding, apparently as a result of loss in receptor affinity, was observed immediately after cell lysis and before separation of cell fractions. This appears to be the result of a change in the receptor and/or associated structures which takes place immediately after cell lysis. A smaller shift in receptor affinity was observed during preparation of plasma membrane-enriched fractions from A875 cells. However, the threefold difference in receptor affinity between A875 cells and membranes is more difficult to demonstrate definitively than the tenfold difference between high-affinity receptor on PC12 cells and the homogenous low-affinity receptor on PC12 membranes. Thus, it is not yet clear whether receptor affinity declines during A875 membrane preparation.

Some of the PC12 membrane preparations used in this study did retain a detectable, but significantly reduced amount of the high-affinity form of the receptor. Quantitation of high-affinity receptor on membranes was difficult to determine and was not always detectable, even within the same membrane preparation. At least two possible explanations can be proposed for this phenomenon: (1) the amount of high-affinity receptor remaining in PC12 membranes is near the limit of resolution of the assay used to detect it; (2) the remaining high-affinity receptor may be labile and gradually revert to the low-affinity form. It is not possible to distinguish between these possibilities at this time. Experiments not shown here, however, clearly indicate the complete absence of high-affinity receptor in detergent-solubilized extract from PC12 membranes. The Kd for solubilized receptor obtained from these experiments was approximately 3 nM. Preliminary experiments (not shown) also indicate that receptor in PC12 membranes can be partially reconstituted to a high-affinity state by the addition of highly concentrated PC12 cell cytoplasmic extracts. A similar shift in PC12 receptor affinity does not appear to be induced by the addition of adenosine triphosphate (ATP) or guanosine triphosphate (GTP) to membranes.* In sum, these experiments suggest the presence of a cell component that can associate with NGF receptor in PC12 cells, presumably after binding of NGF, which induces a change in the NGF receptor to a high-affinity, trypsin-resistant state. This cell component is rapidly lost or diluted after cell lysis and is apparently not functionally coupled or is absent in A875 cells. The nature of this purported factor has not been further elucidated; however, a very similar change in receptor properties can be induced by the addition of WGA, as has been described in detail for PC12 cells [8] and membranes [11] and for membranes and detergent-solubilized extracts from A875 cells [11].

It is important to distinguish between receptor present on the cell surface in a high-affinity, trypsin-resistant state and internalized receptor, which would also appear to be trypsin-resistant and nondissociable from NGF. This is not a concern in the case of A875 cells since no high-affinity, trypsin-resistant binding was observed

*(S.E. Buxser and G.L. Johnson, unpublished observation)

nor do A875 cells internalize significant amounts of ^{125}I-NGF. A significant amount of ^{125}I-NGF was taken up by PC12 cells 60 min after a shift in incubation temperature from 0° to 37°C. This was accompanied by an increase in the amount of TCA-soluble ^{125}I, indicating that degradation of ^{125}I-NGF was taking place after endocytosis. However, since trypsin-resistant ^{125}I-NGF–NGF-receptor complex was detected on PC12 cells after equilibration of binding at 0°C, receptor-mediated endocytosis cannot account for all of the trypsin-resistant complex observed.

In summary, NGF receptor in PC12 cells has properties different from the properties of NGF receptor in A875 cells. That is, a portion of the NGF receptor in PC12 cells is present or can be converted by NGF to a high-affinity, trypsin-resistant state, but A875 cells possess receptor only in a uniform lower-affinity, trypsin-sensitive state. Also, PC12 cells internalize a significant amount of ^{125}I-NGF, but A875 cells do not. Rather than being the result of substantial differences between the receptor on the two cell types, the evidence provided here supports the hypothesis that NGF receptor in intact PC12 cells is regulated differently from NGF receptor in intact A875 cells. The data obtained by covalent crosslinking of ^{125}I-NGF to NGF receptor clearly indicate that no gross structural differences exist between NGF receptor on PC12 or A875 cells. Additionally, a shift in affinity was observed when either PC12 or A875 cells were lysed and enriched plasma membrane fractions were prepared. After cell lysis NGF receptor from either cell reverted to a homogenous, low-affinity state, which readily reverted to a high-affinity state in the presence of WGA. Thus, the NGF receptor present in either plasma membrane-enriched fraction not only maintained structural integrity as evidenced by an unchanged covalent crosslinking pattern, but could still be converted to the high-affinity state. We conclude that the differences in NGF receptor properties observed for receptor on PC12 and A875 cells are the result of differences in the respective intracellular environments and are not a consequence of significant structural differences in the receptor molecules. Thus, we have obtained evidence that strongly indicates that the binding units of the NGF receptor from PC12 and A875 cells have significant structural homology. Our data imply that the differences in receptor properties described above between the two cell types are related to cell components other than the NGF binding unit of the receptor. These differences may relate to, as yet, unidentified subunits of the receptor or cell components independent of the receptor. Our recent purification of the NGF receptor [17] should allow us to address these questions.

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