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The internalization of nerve growth factor by high-affinity receptors on pheochromocytoma PC12 cells

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The internalization and subsequent fate of the two populations of nerve growth factor (NGF) receptors on pheochromocytoma PC12 cells were explored either by identifying the relative amounts and sizes of the receptors, after incubation of cells with [^{125}I]NGF, by cross-linking with a photoreactive heterobifunctional reagent or by following the topological distribution of the cross-linked receptors with time. The ratio of the slow, high-affinity to the fast, low-affinity NGF receptor decreased over a 5-h incubation with [^{125}I]NGF in a process which did not involve proteolytic conversion of the slow to the fast receptor. During this period the cross-linked slow receptor moved from a trypsin-labile to a trypsin-stable site suggestive of internalization. In contrast, the cross-linked fast NGF receptor remained trypsin sensitive for at least 2 h of incubation, indicative of a constant cell surface localization. The internalized [^{125}I]NGF in the cross-linked slow NGF receptor was not degraded, indicating that cross-linking, by preventing the acid pH-induced dissociation of the NGF–receptor complex in the endosomes, blocks normal sorting of [^{125}I]NGF to the lysosomes. The cross-linked receptor was not recycled to the cell surface. If this reflects the properties of the unmodified receptor then another process, possibly receptor conversion, is required to replenish slow NGF receptors in the cell surface.

Key words: internalization/nerve growth factor/pheochromocytoma PC12/receptors/recycling

Introduction

Since it is well established that nerve growth factor (NGF), a protein that mediates the development, maintenance and regrowth of sympathetic and some sensory neurons (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980; Campenot, 1982a,b) is synthesized in peripheral targets (Korsching and Thoenen, 1983a; Heumann *et al.*, 1984a; Shelton and Reichardt, 1984) there must exist some mechanism for conveying NGF-induced signals from the nerve terminals to the neuronal cell bodies where gene expression is controlled. The finding that [^{125}I]NGF is transported from peripheral target tissues to the neuronal cell body (Hendry *et al.*, 1974) suggested that NGF itself may function in some way as the retrograde messenger. Convincing proof of the retrograde transport of target-derived endogenous NGF in axons comes from experiments showing accumulation of NGF on the distal side of a crush in rat sciatic nerve (Korsching and Thoenen, 1983b). Retrograde transport of NGF requires a specific interaction with membrane receptors at the nerve terminal (Dumas *et al.*, 1979; Schwab *et al.*, 1982) and the subsequent transport of the endocytosed NGF receptor-containing vesicles along microtubules (Schnapp *et al.*, 1985). NGF arrives at the cell body in an intact form (Johnson *et al.*, 1978; Dumas *et al.*, 1979) but is subsequently degraded at a quite high rate in lysosomal structures (Heumann *et al.*, 1984b; Korsching and Thoenen, 1985). Both the mechanism of this retrograde transport, as well as other studies (Heumann *et al.*, 1981; Seeley *et al.*, 1983), emphasize that the intracellular signals are not derived from NGF in the cytoplasm of the neuron.

Two types of *in vivo* transport processes for NGF have been identified (Dumas *et al.*, 1979): one of high affinity and low capacity and another of lower affinity but higher capacity. These two saturable processes may correspond to transport systems initiated by the binding of NGF to the two populations of NGF receptors with high and low affinity which have been identified on sympathetic and sensory neurons by equilibrium and kinetic binding experiments (Sutter *et al.*, 1979; Godfrey and Shooter, 1986). The NGF-responsive PC12 rat pheochromocytoma cell line also possesses two populations of NGF receptors with similar characteristics to those on primary neurons (Yankner and Shooter, 1979; Schechter and Bothwell, 1981). Since these cells readily internalize NGF at physiological temperatures (Rohrer *et al.*, 1982; Bernd and Greene, 1983) they provide a model system for exploring this aspect of NGF's action in detail. Internalization of NGF by PC12 cells has all the characteristics of receptor-mediated endocytosis (Layer and Shooter, 1983) in that it is inhibited by low temperature and lysosomotropic agents. It occurs over a wide range of NGF concentrations (0.1–6 nM) although the capacity of the cells to internalize, as judged by the amount of [^{125}I]NGF degraded in a given time, is proportionately less at the higher concentrations (Gunning *et al.*, 1981; Layer and Shooter, 1983). Internalization via the higher affinity receptors was demonstrated by removing low-affinity receptors and their bound NGF by limited trypsin digestion after maximum NGF binding is attained and noting the subsequent significant decrease in high-affinity receptors over the next several hours (Shooter *et al.*, 1981). Using a low-pH, high-salt procedure to differentiate between surface-bound and internalized NGF, Bernd and Greene (1984) noted that the concentration dependence of NGF internalization is similar to that of NGF binding to the high-affinity receptors on PC12 cells. Interestingly, the amount of NGF internalized is considerably greater than that anticipated from the number of high-affinity NGF receptors indicating that, if internalization is restricted to this population of receptors, the latter must be replaced continuously either by recycling or receptor conversion or synthesis (Rohrer *et al.*, 1982; Bernd and Greene, 1984).

In concurrent research we have examined the process of NGF internalization and NGF receptor recycling by taking advantage of the different sizes and trypsin sensitivity of the two PC12 NGF receptors. Photoaffinity cross-linking experiments with a heterobifunctional agent show that the high-affinity, slowly dissociating (slow) NGF receptor complex has a mol. wt of 158 000 while the low-affinity, rapidly dissociating (fast) NGF receptor complex has a mol. wt of 100 000 (Hosang and Shooter, 1985). Mild tryp-

sinization reduces the size of the slow NGF receptor complex on the external plasma membrane of the cell by ~10 000 daltons while completely degrading the fast NGF receptor complex. This methodology has been used used to show that the slow NGF receptor complex is preferentially internalized but that neither recycling to the surface membrane nor significant processing of the cross-linked slow NGF receptor complex is observed.

Results

Only the M_r 158 000 species disappears after prolonged incubation at 37°C

It has been previously demonstrated that the two kinetically distinct types of NGF receptors (slow and fast) present in PC12 cells are also physically different: an M_r 158 000 band was identified as the cross-linked [^{125}I]NGF–slow receptor complex and an M_r 100 000 band as the cross-linked [^{125}I]NGF–fast receptor complex (Hosang and Shooter, 1985).

To investigate whether the two types of receptor behave differently with regard to internalization, PC12 cells were first loaded with 0.8 nM [^{125}I]NGF in the cold, then incubated for various periods of time at 37°C and finally subjected to cross-linking with hydroxysuccinimidyl-4-azidobenzoate (HSAB). Figure 1A shows that the M_r 158 000 and M_r 100 000 species were labeled to approximately equal extents when cross-linking was performed, under the conditions described, immediately after incubation on ice. Upon incubation at 37°C, however, the amount of the labeled M_r 158 000 species gradually decreased so that after 5 h it was significantly less than in the control. The labeling in the M_r 100 000 band also decreased in the sense that less label was apparent in the trailing edge of the band with time. However, in addition, a time-dependent increase in the labeling of a band with M_r 85 000 was observed.

Essentially the same result was obtained when, as shown in Figure 2A, incubation of PC12 cells with [^{125}I]NGF was performed entirely at 37°C without prior incubation on ice. After incubation with 0.8 nM [^{125}I]NGF at 37°C for 30 min prior to cross-linking the two M_r 158 000 and 100 000 complexes were observed in approximately equal amounts and a substantial fraction of the M_r 85 000 species was also present (Figure 2A, lane 1). When the incubation period was prolonged to 5 h most of the M_r 158 000 complex disappeared but little change was observed in the M_r 100 000 or 85 000 regions (Figure 2A, lane 2).

To investigate whether the decreased labeling of the M_r 158 000 complex after prolonged incubation at 37°C was due to its transformation, by limited proteolysis, into one of the species in the M_r 85 000–100 000 region, the above experiment was repeated at an [^{125}I]NGF concentration (0.1 nM) where binding and cross-linking produced mainly the M_r 158 000 complex (Hosang and Shooter, 1985; Figure 2B, lane 3). Again after a 5-h incubation with [^{125}I]NGF prior to cross-linking, little of the M_r 158 000 labeled complex was observed (Figure 2B, lane 4). However, the decrease in M_r 158 000 species was not accompanied by an appearance of labeled material in either the M_r 100 000 or 85 000 range. This result shows that the disappearance of the M_r 158 000 species is not a result of conversion to the lower mol. wt species and in turn suggests that the M_r 85 000 component is derived by proteolysis from the M_r 100 000 complex. A possible explanation for the selective decrease in the labeling of the M_r 158 000 slow receptor complex after prolonged incubation at 37°C is that this complex, but not the smaller M_r 100 000 fast receptor complex, is internalized and hence unavailable for cross-linking.

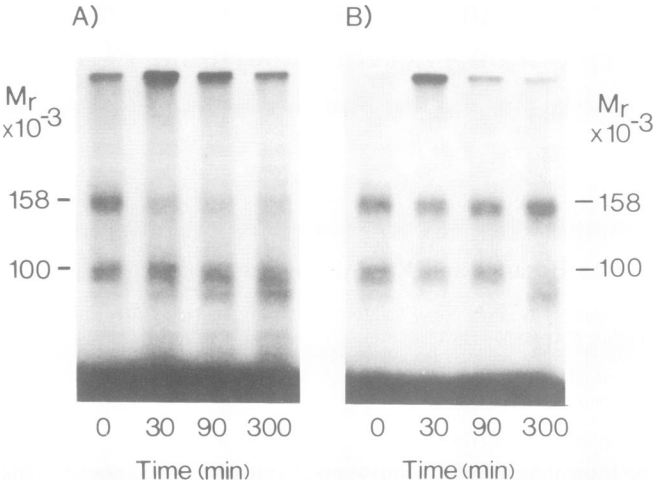

Fig. 1. (A) Influence of prolonged incubation at 37°C on the labeling pattern of PC12 cells pre-equilibrated with [^{125}I]NGF in the cold. PC12 cells were incubated in 10 mM Hepes/Krebs–Ringer medium, pH 7.35, containing 1 mg/ml BSA, in the presence of 0.8 nM [^{125}I]NGF for 2 h on ice. After shifting the temperature to 37°C, incubation was continued for the times indicated. Cells were then cooled to 0.5°C and cross-linking was performed with 50 μM HSAB as described in Materials and methods. Following washing by dilution into excess ice-cold Tris–saline, pH 7.0 and centrifugation, the cells were solubilized by boiling for 5 min in 1% SDS in the presence of 50 mM dithiothreitol. Cell proteins (100 μg samples) were subjected to electrophoresis on 6% polyacrylamide slab gels. Shown is an autoradiogram of the stained, dried gel, which was obtained after an 8-day exposure. The extrapolated mol. wt values of the labeled bands are indicated on the left (**A**) or right (**B**). **(B)** The internalized M_r 158 000 (cross-linked) NGF–receptor complex is not degraded rapidly. PC12 cells were incubated for 2 h on ice with 0.8 nM [^{125}I]NGF and then subjected to cross-linking with 50 μM HSAB as described in Materials and methods. After repeated washing by dilution into 10 mM Tris–saline, pH 7.0 and centrifugation, the photolysed cells were resuspended in fresh Hepes/Krebs–Ringer medium and incubated for the times indicated at 37°C. Following another washing cycle by dilution into Tris–saline and centrifugation, the cells were then solubilized and analysed on 6% SDS–polyacrylamide gel in the presence of 50 mM dithiothreitol. Shown is an autoradiogram of the dried, fixed gel.

One test for the internalization of the M_r 158 000 complex is based on the known down-regulation of NGF receptors in PC12 cells during incubation with ligand (Layer and Shooter, 1983). The results in Figure 2C show that when PC12 cells were pre-incubated with 1.0 nM unlabeled NGF for 5 h at 37°C, and then washed extensively at the same temperature to remove the NGF from the receptors (Landreth and Shooter, 1980) prior to determining the surface receptor composition by the cross-linking method, a significant decrease in the M_r 158 000 complex was observed (Figure 2C, lane 6). Since the same result was obtained when [^{125}I]NGF was used in the pre-incubation (experiment in Figure 2B) it is unlikely that occupancy of the M_r 158 000 complex by unlabeled NGF, not removed in the washing procedure, accounts for the decreased labeling of this species.

These experiments emphasize that there is a significant difference in the behavior of the slow and fast NGF receptors when PC12 cells are exposed at 37°C to NGF for several hours. Binding to the fast receptor remains relatively constant with time although an increasing proportion of a lower molecular form of this receptor complex (M_r 85 000) appears. In contrast binding to the slow receptor decreases with time and is especially marked after 5 h, suggestive of a loss of these receptors from the external cell surface. Interestingly, if the slow receptors are replenished in the cell membrane (irrespective of the mechanism) the rate of replacement does not, at least after a period of 5 h, equal the rate of loss.

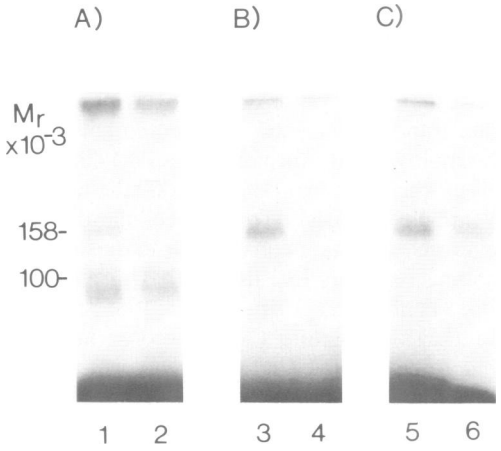

Fig. 2. (A,B) The reduced labeling of the M_r 158 000 complex after prolonged incubation of PC12 cells with [^{125}I]NGF is not due to proteolysis. PC12 cells were incubated at 37°C with 0.8 nM [^{125}I]NGF **(A)** or 0.1 nM [^{125}I]NGF **(B)** for 30 min **(lanes 1** and **3)** or 5 h **(lanes 2** and **4)**. Following a 5-min incubation at 0.5°C, cross-linking was performed with 50 μM HSAB as indicated in Materials and methods. Samples were then subject to electrophoresis on a 6% slab gel as described in the legend to Figure 1. Shown are autoradiograms of the stained, dried gels, obtained after an 8-day exposure **(A)** or a 21-day exposure **(B)**. The extrapolated mol. wt values of the labeled bands are indicated on the left. **(C)** The reduced labeling of the M_r 158 000 complex after prolonged incubation of PC12 cells with [^{125}I]NGF is due to down-regulation. PC12 cells were incubated in 10 mM Hepes/Krebs−Ringer medium, pH 7.35 containing 1 mg/ml BSA, in the presence of 1.0 nM unlabeled NGF for 0 h **(lane 5)** and 5 h **(lane 6)** at 37°C. The cells were then washed by dilution into Tris−saline at 37°C and centrifugation, the process being repeated three times. This was followed by incubation of the cells in the above medium but containing 0.1 nM [^{125}I]NGF for 1 h on ice and cross-linking with 50 μM HSAB as described in Materials and methods. Samples were analysed by electrophoresis. Shown is an autoradiogram of the stained dried gel after a 21-day exposure.

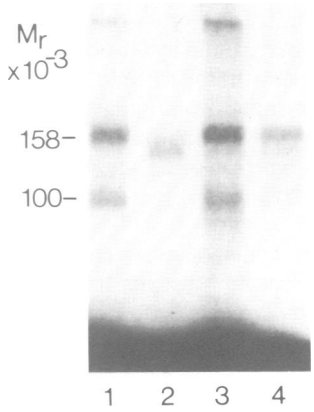

Fig. 3. Incubation at 37°C following cross-linking confers trypsin-insensitivity to the M_r 158 000 (slow) receptor complex only. PC12 cells incubated with 0.8 nM [^{125}I]NGF for 60 min at 23°C were subjected to cross-linking with 0.5 μM HSAB as described in Materials and methods. After washing twice by dilution into 10 mM Tris−saline, pH 7.0 and centrifugation, cells were resuspended in fresh 10 mM Hepes/Krebs−Ringer medium and incubated for 15 min **(lanes 1,2)** or 120 min **(lanes 3,4)** prior to treatment with **(lanes 2,4)** or without **(lanes 1,3)** trypsin (50 μg/ml) for 15 min at 37°C. Trypsinization was stopped by cooling and repeated washing by centrifugation and resuspension in Tris-buffered saline. The final cell pellets were solubilized and analysed by electrophoresis on 6% polyacrylamide slab as described in the legend to Figure 1. An autogradioagram of the stained, dried gel was obtained after a 14-day exposure. The extrapolated mol. wt values of the labeled bands are indicated on the left.

Only the M_r 158 000 cross-linked NGF−receptor complex is protected from the action of trypsin

The differential susceptibility of the NGF−receptor complexes to mild trypsinization provides a tool for monitoring when the receptor complexes are removed from the external plasma membrane of the PC12 cell (Hosang and Shooter, 1985). Trypsinization completely eliminates the surface NGF−receptor complexes of the fast type (M_r 100 000) while reducing the mol. wt of the surface slow receptor complex (M_r 158 000) by 10 000 without affecting NGF binding.

When, as shown in Figure 3, lane 1, PC12 cells loaded with [^{125}I]NGF (0.8 nM) were subjected to cross-linking in the usual way and subsequently incubated in fresh medium for another 15 min at 37°C the labeling pattern did not differ significantly from that seen when cells were solubilized immediately after cross-linking (Figure 1A, 0 time). Similarly when the [^{125}I]NGF loaded and cross-linked cells were subjected to trypsin action on ice (Figure 3, lane 2) the results were the same as if the trypsin treatment had preceded rather than followed the cross-linking reaction (Hosang and Shooter, 1985), namely the M_r 158 000 complex was processed to the M_r 148 000 complex and the M_r 100 000 complex was abolished. This experiment shows that after a 15-min incubation at 37°C the majority of both types of cross-linked receptor complexes are still in the outer surface of the cells and available to trypsin. Continuing the incubation of the cross-linked cells for a further period of 2 h did not substantially alter the labeling pattern (Figure 3, lane 3). However, when these cells were exposed to trypsin the size of the M_r 158 000 complex did not change (Figure 3, lane 4) showing that the complex was not available to trypsin action and therefore was located within the cell. The failure to detect any significant amount of the M_r 148 000 complex also suggests that little if any recycling of this receptor to the cell surface occurs within 2 h. In contrast the M_r 100 000 complex was almost completely degraded showing that most of it remained in the outer cell surface. The same result was obtained when the HSAB concentration was reduced to 0.5 μM suggesting that the inability of the M_r 100 000 cross-linked complex to be internalized is not due to interference of the bifunctional cross-linker with the internalization process for this receptor.

The internalized cross-linked M_r 158 000 NGF−receptor complex is not degraded rapidly

Continued incubation at 37°C of the cross-linked M_r 158 000 complex did not result in the decrease seen with the slow receptor species to which [^{125}I]NGF was not cross-linked (Figure 3). In order to determine the fate of the internalized, cross-linked M_r 158 000 complex, the duration of the incubation after cross-linking was prolonged. The data in Figure 1B show that there was no detectable loss or proteolytic processing of the internalized M_r 158 000 complex during 90 min of incubation. Only when the incubation was extended to 5 h was a trace of a complex of slightly lower mol. wt than 158 000 observed. Similarly the M_r 100 000 complex, still facing the external milieu of the PC12 cells, was not degraded during the first 90 min of incubation at 37°C. However, after 5 h incubation this complex was almost quantitatively converted to the species of M_r 85 000. In comparing the results in Figure 1A and B it is clear that the cross-linked M_r 100 000 complex was degraded more slowly during 90 min of incubation than the M_r 100 000 complex which was not cross-linked before the incubation. In the latter instance the appearance of the M_r 85 000 complex was just evident after

30 min of incubation and quite prominent after 90 min. However, once the processing of the cross-linked M_r 100 000 complex began (sometime after 90 min incubation) it apparently proceeded faster than the processing of the uncross-linked complex. It is not possible to determine from the present data whether or not the processing of the internalized, cross-linked M_r 158 000 complex differs from that of an uncross-linked complex.

Discussion

Receptor-mediated endocytosis is characteristic of a number of growth factor receptor systems including NGF (Layer and Shooter, 1983), epidermal growth factor (EGF, Schlessinger *et al.*, 1983) and insulin (Kahn, 1976). Whether endocytosis itself plays a role in signal transduction or not remains unclear, however.

Two experimental protocols have been used in this work to monitor the fate of the two populations of NGF receptors on PC12 cells after binding NGF. In the first, the disappearance of the NGF−receptor complexes from the cell surface or their modification was followed by cross-linking the complexes with HSAB at different times after the start of the incubation and analysing the radiolabeled protein species by gel electrophoresis. In the second, a more direct but also more invasive procedure, the NGF−receptor complexes were cross-linked prior to further incubation. In this instance the differential trypsin sensitivity of the two complexes was also used to determine the topological distribution of the receptor complexes.

Although the photoaffinity cross-linking procedure is not quantitative, it is clear that the ratio of slow to fast NGF receptors on the cell surface (identified as their M_r 158 000 and 100 000 complexes, respectively) decreases markedly over a 5-h incubation with NGF and that this is not due to a conversion of the slow receptor to a species of lower mol. wt. The finding that the cross-linked M_r 158 000 complex moves from a trypsin-sensitive to a trypsin-protected site after 2 h incubation suggests that the loss of surface slow receptors is due to internalization. In contrast, the cross-linked M_r 100 000 fast NGF−receptor complex remains trypsin-labile even after a 2 h incubation, consistent with its continued cell surface localization. An alternative explanation for this last result is that the cross-linking procedure itself interferes with the internalization of the fast NGF receptor. If this is true then it must also occur even at very low concentrations of the cross-linking agent and, furthermore, be unique to the fast receptor. It is worth noting that because of the low cross-linking efficiency of HSAB (only ~5% of the NGF receptor complexes are covalently cross-linked; Hosang and Shooter, 1985) it is not possible to address the question of the possible biological function of a cross-linked receptor.

The conclusion from these experiments, that the slow NGF receptor is preferentially internalized, agrees with the data showing down-regulation of slow receptors on PC12 cells stripped of fast receptors by trypsinization (Shooter *et al.*, 1981) and a similarity between the concentration dependence of internalization and binding to the slow, but not the fast, receptor (Bernd and Greene, 1984). In line with this conclusion are the observations that neither PC12 mutants with no or low numbers of slow receptors (Green *et al.*, 1986), nor the human melanoma cell line A875 which possesses only fast NGF receptors (R.D. Vale, J.Rubenstein and E.M.Shooter, unpublished data) internalize NGF. Evidence has recently been presented that, similarly, interleukin-2 bound to high-affinity receptors is internalized whereas occupied low-affinity receptors do not mediate internalization of interleukin-2 (Weissman *et al.*, 1986).

The structure of the rat fast NGF receptor has recently been determined by gene transfer and molecular cloning (Radeke *et al.*, 1987). It comprises a 396-residue peptide chain with a single membrane-spanning region separating extracellular (222 amino acid residues) and cytoplasmic (151 amino acid residues) domains. The mol. wt of this protein core is 42 478 and since the mature receptor has a mol. wt of ~82 000 the fast NGF receptor is very carbohydrate rich. The extracellular domain contains four cysteine-rich repeating units similar to those found in other peptide-binding receptors like the EGF (Ullrich *et al.*, 1984), insulin (Ebina *et al.*, 1985; Ullrich *et al.*, 1985) and low-density lipoprotein (LDL) receptors (Yamamoto *et al.*, 1984). The cytoplasmic domain is too short to encompass an endogenous kinase activity and in keeping with this lacks the ATP binding site characteristic of kinases (Kamps *et al.*, 1985). The cDNA for this fast NGF receptor hybridizes to a single 3.7-kb mRNA in PC12 cells and sensory neurons, suggesting either that the two NGF receptors in these cells are coded for by different genes or, more likely, that the slow NGF receptor contains the fast NGF receptor as its NGF-binding subunit and an additional membrane protein of ~60 000 mol. wt (Radeke *et al.*, 1987). On this basis both signal transduction and internalization would be mediated by this second protein. A particularly attractive candidate for this protein is the product of the c-*src* proto-oncogene, a 60 000-dalton, plasma membrane phosphoprotein ($pp60^{c\text{-}src}$) with tyrosine-specific kinase activity (Bishop, 1983). The involvement of such a protein would provide a possible mechanism for the NGF-induced phosphorylation of a number of proteins on tyrosine (Halegoua and Patrick, 1980), either in the membrane or, following slow NGF receptor internalization, in Golgi-like structure or the perinuclear region of the cell. Such a dual subcellular location of $pp60^{c\text{-}src}$ has already been demonstrated (Resh and Erikson, 1985).

It is reasonable to assume that internalization of the slow NGF receptor complex is mediated through coated pits (Goldstein *et al.*, 1979), especially since the number of 60−130-nm coated pits on the PC12 cell surface increases 3-fold within 3 min of addition of NGF (Connolly *et al.*, 1981) and that by this mechanism the receptor complex is rapidly delivered to the peripheral and/or perinuclear pre-lysosomes, the endosomes (Mellman *et al.*, 1986). The presence of internalized [^{125}I]NGF in such structures in PC12 cells and sympathetic neurons has been confirmed by electron microscopic autoradiography (Schwab and Thoenen, 1977; Claude *et al.*, 1982; Hogue-Angeletti *et al.*, 1982; Rohrer *et al.*, 1982; Bernd and Greene, 1983). Does the subsequent fate of the NGF or of the slow NGF receptor provide clues to the generation of potential intracellular signals other than those already discussed above? The slow NGF receptor belongs to that class of receptors whose affinity for their ligands is reversibly altered by pH (Mellman *et al.*, 1986). Maximum binding of NGF to this receptor occurs at pH 7.5 close to the pH of the extracellular environment. Binding decreases in mildly acid pH to reach a level, at pH 6.0, which is only ~25% of the maximum (Vale and Shooter, 1984). As a consequence the NGF−receptor complex will largely dissociate in the mildly acidic environment of the endosome (Tycko and Maxfield, 1982) leaving the two components of the complex to be handled separately. The NGF becomes part of the fluid phase of the endosome and there is good evidence from both morphological (Claude *et al.*, 1982; Rohrer *et al.*, 1982; Bernd and Greene 1983) and biochemical studies (Chandler and Herschmann, 1983; Layer and Shooter, 1983; Korsching and Thoenen, 1985) that it is delivered to the lysosomes and degraded. That this pathway

does not produce intracellular signals for the action of NGF is clear from the findings that inhibition of lysosomal NGF degradation by acidotropic agents (Yankner and Shooter, 1979; Layer and Shooter, 1983) or protease inhibitors (Heumann *et al.*, 1984b) has no effect on NGF-induced neurite outgrowth or choline acetyltransferase activity (Heumann *et al.*, 1984b). Correspondingly, injection of NGF antibodies into PC12 cells, to neutralize either native or degraded (but still immunoreactive) NGF in the cytoplasm, has no effect on NGF-induced neurite outgrowth (Heumann *et al.*, 1981; Seeley *et al.*, 1983) or choline acetyltransferase activity (Heumann *et al.*, 1984b). If the slow NGF receptor behaves like most of the other receptors of its class it would rapidly (i.e. within 15 min) recycle to the plasma membrane, bind fresh NGF and repeat the cycle (Brown *et al.*, 1983; Mellman *et al.*, 1986). Examples of peptide-binding receptors which undergo recycling are the insulin receptors on rat hepatocytes (Fehlmann *et al.*, 1982) and insulin receptors on rat adipocytes (Marshall, 1985). Originally, the EGF receptor appeared to be an exception to the recycling pathway (King *et al.*, 1980). Recently, however, the majority of the EGF receptors which had internalized in fibroblasts in response to EGF were found to recycle to the surface after EGF withdrawal, with a $t_{1/2}$ for the recycling of ~15 min (Murthy *et al.*, 1986). Equally interesting is the demonstration that the perinuclear/nuclear envelope region is one station of the intracellular itinerary of the EGF receptor kinase (Murthy *et al.*, 1986), leaving that compartment as a possible place of intracellular message generation. In the present study it was apparent that [^{125}I]NGF in the cross-linked, slow NGF receptor was not degraded over 5 h, nor was the cross-linked receptor complex recycled to the plasma membrane over a 2-h period. In these respects the behavior of the slow NGF receptor is clearly different from that of the insulin and EGF receptor. Cross-linked insulin or insulin analog–receptor complexes are degraded in adipocytes, the latter at the same rate as the dissociable receptor complexes (Reed, 1983; Berhanu *et al.*, 1983). Also, another cross-linked insulin analog–receptor complex still recycles to the plasma membrane, albeit at a relatively slow rate (Fehlmann *et al.*, 1982). The failure of the cross-linked slow NGF receptor to recycle may, therefore, be interpreted in different ways. Either the cross-linking and/or the subsequent washing procedure with buffer containing Tris (Marshall, 1985) interferes with recycling processes as it does with the lysosomal sorting of NGF or the behavior of the cross-linked NGF receptor reflects the properties of the unmodified slow NGF receptor, i.e. it does not recycle or recycles slowly. This last interpretation is supported by the observation that many more uncross-linked slow NGF receptors are internalized over a 5-h period than are replaced. An alternative mechanism to receptor recycling for slow NGF receptor replenishment must, therefore, be considered to account for the large amounts of NGF which are internalized via the slow NGF receptor. One such mechanism has been proposed, namely the conversion, induced by NGF binding of fast to slow NGF receptors (Landreth and Shooter, 1980). This is a particularly attractive proposal because it would account for the appearance of the two types of retrograde transport process observed *in vivo* (Dumas *et al.*, 1979) even though internalization is achieved only by one receptor. Provided the rate of receptor conversion was significantly faster than binding to the two receptors then the characteristics of NGF internalization (and its subsequent transport) would reflect the binding properties of both receptors.

It should be noted that these experiments do not provide evidence for a proteolytic conversion of the larger, slow to the smaller, fast NGF receptor as has been suggested (Massagué *et al.*, 1981). Interestingly the fast NGF receptor does not show the same decrease in binding of NGF at mild acid pH as does the slow receptor (Vale and Shooter, 1984). It follows that if the fast receptor was internalized, the NGF–receptor complex would not dissociate in the endosomal compartments. Perhaps this is the basis for the preferential internalization on the slow NGF receptor.

Materials and methods

NGF (the βNGF subunit) was prepared from adult, male mouse submaxillary gland as previously described (Burton *et al.*, 1978). NGF was radioiodinated using lactoperoxidase to a specific activity of 50–70 c.p.m./pg according to Sutter *et al.* (1979) and was used within 3 weeks. Trypsin was obtained from Worthington and HSAB was from Pierce Chemical Co. Electrophoresis reagents and mol. wt standards were obtained from BioRad. All other chemicals were of the highest quality available.

Cells

PC12 cells (obtained from D.Schubert, Salk Institute, La Jolla, CA) were cultured in H-21 DMEM (GIBCO, Grand Island, NY) supplementd with 10% fetal calf serum (FCS, Irvine Scientific, Irvine, CA or GIBCO) and 5% horse serum (GIBCO) as described earlier (Hosang and Shooter, 1985).

Affinity labeling protocol

Affinity labeling with HSAB was performed as previously described (Hosang and Shooter, 1985). Briefly, PC12 cells were first washed on the dish with 10 mM Hepes/Krebs–Ringer saline, pH 7.35, containing 1 mg/ml each of D-glucose and bovine serum albumin (binding medium) and then harvested by trituration. Cells were then washed one additional time by centrifugation in the same medium and were finally resuspended at 2×10^6 cells/ml. Samples (1 ml) of cells were then incubated with [^{125}I]NGF under the conditions specified in each experiment. Without further washing, HSAB, freshly dissolved in dimethylsulfoxide, was added and the mixture incubated for 3 min in the dark on ice and then photolysed for 7 min in a quartz cuvette in the cold with a 200 W mercury Oriel high-pressure arc lamp equipped with a 330 nm cut-off filter. Photolysis was stopped and cells were diluted into excess Tris–saline, pH 7.0, and pelleted by centrifugation at 1000 *g* for 5 min. After washing one additional time, the cells were either resuspended in fresh binding medium and used for internalization experiments or immediately solubilized by boiling for 5 min in sample buffer (58 mM Tris–HCl, pH 6.8, 10% glycerol, 1% SDS and 0.005% bromophenol blue) containing 50 mM dithiothreitol. Samples were stored at −20°C until analysed by SDS–polyacrylamide gel electrophoresis.

SDS–polyacrylamide gel electrophoresis and autoradiography

Samples processed as described above were thawed, centrifuged at 10 000 *g* for 10 min in the cold and 100 μg of total cell protein were loaded per lane of a 1.5 mm thick polyacrylamide slab gel. Gel electrophoresis was performed according to Laemmli (1970), using a 3% stacking gel and a 6% separating gel. After electrophoresis the gel was stained in 0.05% Coomassie blue, 25% 2-propanol, 10% acetic acid and destained in 10% methanol and 10% acetic acid. Autoradiograms were obtained from the dried gel after exposure for the indicated length of time to Kodak X-Omat R film, using a Du Pont Lighting Plus enhancing screen. Molecular weight standards used in a parallel run were: myosin (M_r 200 000), β-galactosidase (M_r 116 000), phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 45 000) and carbonic anhydrase (M_r 31 000).

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