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Ultrastructural Studies on the Intracellular Fate of ^{125}I-Nerve Growth Factor in Cultured Rat Sympathetic Neurons

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Primary cell cultures of sympathetic neurons from rat were exposed to ^{125}I-nerve growth factor (NGF) and the fate of the NGF in the cell was followed using electron microscopic autoradiography. The intracellular localization of NGF was determined in the cell bodies and in the proximal neurites of neurons that had been grown in three-chamber dishes, following 5 or 24 hr of retrograde transport of NGF from the distal portions of the neurites. Label in the proximal neurites was predominantly associated with lysosomes and multivesicular bodies (MVBs), and at 5 hr elongated tubular elements were especially heavily labeled. Most of the label in the cell bodies was concentrated in lysosomes and MVBs. Lysosomes accounted for the largest fraction (45–60%) of the grains in the cell body, with a labeling density (LD = % grains/% area) of 3–5, while MVBs accounted for 5–10% of the grains with an LD of 5–20. We observed no evidence of nuclear labeling after 5 or 24 hr of retrograde transport. Mass cultures of neurons were incubated for 22 hr with NGF in the presence of the lysosomal inhibitors chloroquine (CQ, 0.05 mM) or methylamine (MA, 10 mM). In both agents the lysosomes were swollen with membranous material but still sequestered NGF, especially in CQ where the lysosomes were associated with almost 65% of the grains and had an LD of 6. CQ and MA had different effects on the morphology of the MVBs: in CQ they were few in number and compact while in MA they were numerous and appeared swollen and vacuolated. We observed no evidence for the nuclear accumulation of NGF even in the presence of the lysosomotropic agents.

Key words: nerve growth factor, sympathetic neurons, electron microscopic autoradiography, retrograde axonal transport, lysosomotropic agents, internalization of nerve growth factor

Nerve growth factor (NGF) is a potent trophic agent in the sympathetic nervous system: Postganglionic sympathetic neurons are dependent on NGF for survival and differentiation during development, and remain responsive to NGF throughout life [1]. To understand the mechanism of action of NGF, we need to know the fate of NGF as it interacts with and influences responsive cells. Several studies aimed at

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detecting and characterizing such interactions have been carried out, with the following results.

Sympathetic neurons of several species have high affinity, saturable plasma membrane receptors for NGF; the binding affinity of these receptors is of the same magnitude as the half-maximal dose for biological response [2,3]. Following the binding of NGF to plasma membrane receptors, specifically bound NGF is internalized [3,4].

Sympathetic neurons in vivo can transport NGF from the periphery to the cell body via retrograde axonal transport [5–7]. Recently the same phenomenon has been demonstrated in an in vitro system in which the concentration of NGF and other parameters can be more accurately measured and where no other cell types are present to confound the results [3]. In this situation, only the peripheral neurites of the neurons, not the cell bodies, need be in contact with NGF for the cells to survive [8]. This suggests that the transported NGF may play a role in the survival of the cells.

Ultrastructural studies, in vivo [9,10] and in vitro [3,4], have shown that much of the NGF that is internalized and transported by sympathetic neurons becomes concentrated in membrane-limited bodies, especially secondary lysosomes. While much of the NGF is associated with conventional lysosomes, multivesicular bodies (MVBs) appear to contain the highest concentrations of NGF [3,9,10].

Experiments using related cell types have suggested an association of NGF with nuclear structures. Biochemical studies using cell fractionation of embryonic sensory neurons have suggested an association of NGF with the nucleus [11], and similar studies of pheochromocytoma (PC-12) cells have suggested, more specifically, an association with the nuclear envelope [12,13]. While evidence from a light microscopic immunocytochemical study of PC-12 cells suggested that NGF accumulated in the nucleus [14], a similar study of sympathetic neurons in vitro did not [15]. Several ultrastructural studies of intact neurons in vivo [9,10,16,17] or in vitro [3,4] have failed to demonstrate a nuclear concentration of NGF. These differences have not yet been resolved.

It will be important to know to what extent binding, internalization, retrograde transport, lysosomal degradation, and association with the nucleus are important in the biological effects of NGF. The work presented in this paper represents an attempt to characterize in more detail the fate of NGF within responsive cells. Using cultured sympathetic neurons, we have examined the intraaxonal organelles responsible for the retrograde transport of ^{125}I-NGF to the cell body, and the fate of the NGF once it arrives there. In the cell bodies at both five and 24 hr of retrograde transport, the label was concentrated in lysosomes and MVBs, and there was no evidence for the preferential accumulation of NGF in any nuclear structures. We have also examined the localization of internalized ^{125}I-NGF when the cells were treated with the lysosomotropic agents chloroquine and methylamine and the effects of these agents on the lysosomal organelles are described.

MATERIALS AND METHODS

Cell Culture

Sympathetic neurons from superior cervical ganglia of neonatal rats were dissociated mechanically and grown on collagen-coated plastic coverslips from 10

days to six weeks. The growth medium was Liebowitz L-15 supplemented with 5% rat serum and other additives such as vitamins and cofactors [18]. Cells to be used for the analysis of retrograde transport were plated into the central well of three-compartment dishes [8]; their neurites grew under the partitions into adjacent compartments where the medium could be changed independently of the medium in the central well. The cells used for the retrograde transport study had been grown in culture for approximately four weeks. Conventional "mass" cultures of neurons plated onto plastic coverslips [18] were used for the other experiments.

Nerve Growth Factor

2.5S NGF was prepared from male mouse salivary glands [19] and iodinated using a lactoperoxidase–glucose oxidase system [3]. The ^{125}I-NGF (specific activity of 30 μCi/μg) was greater than 95% pure on the basis of SDS-polyacrylamide gel electrophoresis and retained 100% biological activity as assessed by the survival of dissociated rat sympathetic neurons in culture. The ^{125}I-NGF was used at a concentration of 20–30 ng/ml (1×10^{-9} M). The saturating dose of ^{125}I-NGF for neuronal survival was 4×10^{-9} M, as it was for unlabeled NGF.

Incubations

Cells to be exposed to ^{125}I-NGF were preincubated without NGF for times up to 8 hr before the medium was replaced with medium containing 30 ng/ml ^{125}I-NGF. To assess the magnitude of nonspecific (ie, nonsaturable) binding, we incubated cells with 30 ng/ml ^{125}I-NGF in the presence of a 50- to 100-fold excess of unlabeled NGF. Incubations were terminated by flooding the dish with unlabeled medium.

To measure the retrograde transport of ^{125}I-NGF, the complete medium in the outer wells of the dish was replaced with medium lacking NGF for 8 hr before the introduction of medium containing ^{125}I-NGF. The incubations were terminated as in the mass cultures.

Autoradiography

Cultures were rinsed free of radioactive medium before they were fixed in glutaraldehyde and osmium tetroxide, dehydrated, and embedded in Epon [3]. Sections 70–85 nm thick were coated with a monolayer of photographic emulsion (L4, Ilford), using a flat substrate method [20,21]. Autoradiograms were exposed for two to three months in the cold and developed in Microdol-X developer (Kodak) or by a fine grain development procedure [20]. Grids were stained for 45 sec in 2% ethanolic uranyl acetate and 10 min in 0.4% lead citrate. A photographic montage was made of all the tissues located in each of the grid squares to be analyzed. The radioactivity associated with each subcellular structure or organelle was then determined. When Microdol X development was used, the structures included in a 50% circle [22,23] surrounding each of the grains were scored; in the case of fine grain development, the structures directly underlying the grains were scored. On the same montages, nuclear and cytoplasmic areas were measured using a digitizer (Talos) and fractional areas were calculated by scoring the structures underlying an array of circles on a transparent overlay [23]. The labeling density (LD) for an organelle represents the percentage of total grains associated with that organelle, divided by its fractional area, and is an indication of relative intensity of labeling.

RESULTS

Retrograde Transport

Figure 1 illustrates a group of sympathetic neurons and their proximal neurites within the central well of a three-compartment culture dish [8]. ^{125}I-NGF was added to the compartments containing the peripheral neurites and the neurons were allowed to transport labeled NGF retrogradely to the cell bodies; the cell bodies and the proximal neurites were not directly exposed to ^{125}I-NGF. Since retrogradely transported ^{125}I-NGF is not transported anterogradely by the neurites that project into the opposite chamber [3], any label contained in the proximal neurites represents material that was travelling toward the cell body at the time of fixation.

Cultures were analyzed following 5 or 24 hr of retrograde transport; during this time ^{125}I-NGF was continuously present in the peripheral chambers. Previous experiments have shown that label accumulates progressively in the cell bodies up to about 8 hr, at which time a steady state is reached, presumably when transport of NGF into the cell bodies is balanced by degradation and the excretion of breakdown products into the medium [3]. Therefore at 5 hr the organelles in the cell body are still in the process of saturation, while at 24 hr they are well beyond the establishment of the steady state.

Examples of the autoradiograms used for quantitative analysis are illustrated in Figures 2 and 3. They represent a cell body and a bundle of proximal processes following 24 hr of retrograde transport of ^{125}I-NGF.

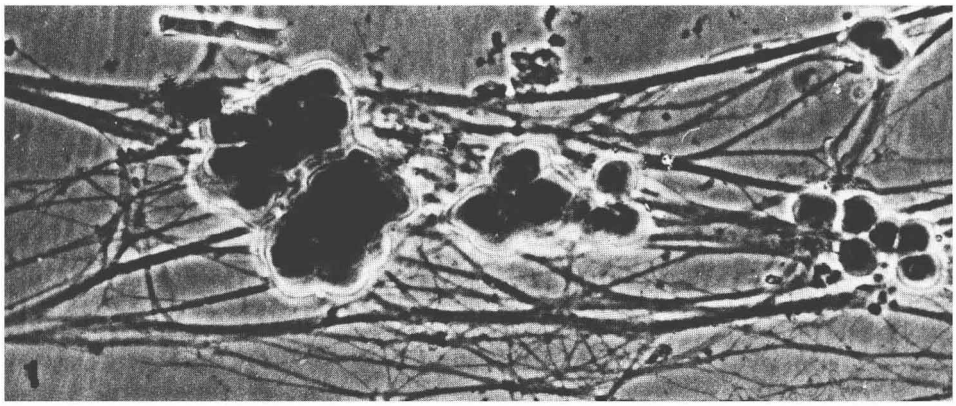
The distribution of label in the cell bodies following five hr of retrograde transport is shown in Table I. Over 40% of the grains were associated with organelles identified as lysosomes on the basis of acid phosphatase staining in similar cultures of sympathetic neurons (unpublished observations). Since lysosomes represented only 16% of the volume of the cell bodies, this demonstrated a significant lysosomal concentration of label, reflected in a labeling density of about 2.7. MVBs had an even higher LD of approximately 5; they were associated with about 9% of the label, but

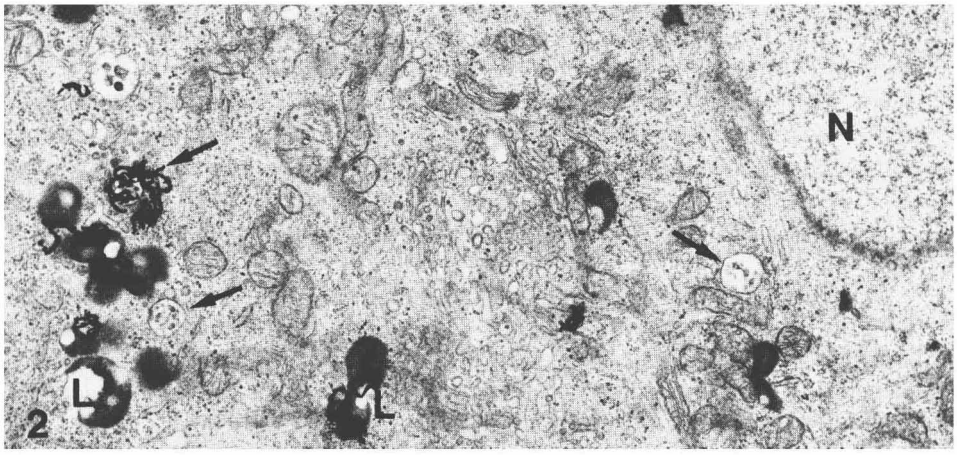
TABLE I. Five Hr of Retrograde Transport: Distribution of ^{125}I-NGF in the Cell Body

	Grains[a] (%)	Fractional area	Labeling density[b]
Nucleus (total)	0.7	15.9	0.04
Nuclear membrane	0	1.2	0
Cytoplasmic organelles			
Lysosomes	44.7	16.7	2.7
MVB	9.2	1.8	5.0
Vesicles	0	1.4	0
Golgi	1.1	3.4	0.3
Mitochondria	10.3	13.3	0.8

[a]Total number of grains scored (fine grain development) = 900. Structures such as microtubules, endoplasmic reticulum or plasma membrane were not preferentially labeled. They are not included here, so the percentages do not add up to 100%.

[b]Labeling density (LD) for each organelle is calculated by dividing the percentage of grains assigned to that organelle by the fractional area of that organelle. If label is concentrated within one class of organelles so that they are assigned a large fraction of the grains, other organelles will have a smaller fraction of the total grains than they would have if the distribution of label were totally random. Therefore some organelles may have an LD of less than 1.





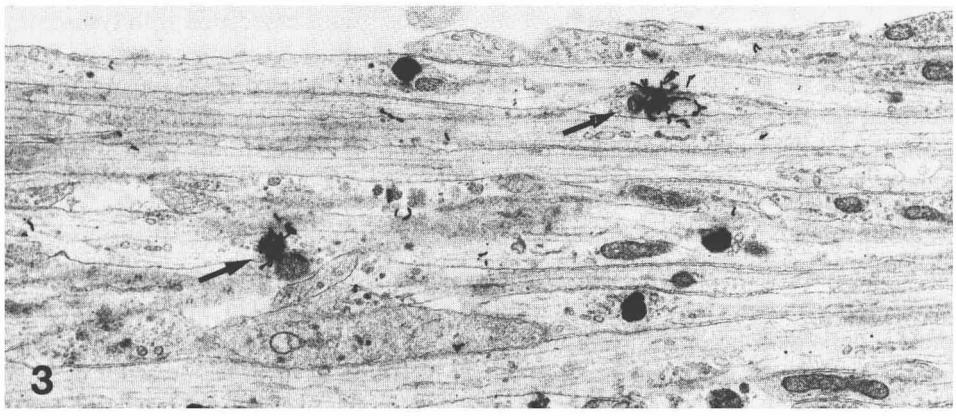


Fig. 1. Phase micrograph of a group of fixed, embedded neuronal cell bodies and their proximal neurites. They were grown for 4 weeks in a three-compartment culture dish. To assess retrograde transport, such cells were exposed to ^{125}I-NGF on their distal neurites, retrograde transport was allowed to proceed for different times, and electron microscopic autoradiograms were prepared for analysis. $\times 175$.

Fig. 2. Electron microscopic autoradiogram of a neuronal cell body that received ^{125}I-NGF for 24 hr via retrograde axonal transport from its peripheral processes. Microdol X development. Arrows indicate multivesicular bodies. L, lysosome; N, nucleus. $\times 12{,}500$.

Fig. 3. Electron microscopic autoradiogram of a bundle of neurites that were in the process of transporting ^{125}I-NGF towards the cell bodies at the time of fixation. Fine grain development. Arrows indicate heavily labeled lysosomes. $\times 14{,}200$.

represented less than 2% of the volume of the cells. Neither the nuclear membrane nor the nucleoplasm, which had a fractional volume comparable to that of the lysosomes, exhibited any preferential labeling. The nucleus contained only 0.7% of the grains, and there were no grains at all in contact with the nuclear membrane. Neither the Golgi apparatus nor the endoplasmic reticulum was preferentially labeled.

The distribution of label in proximal neurites following five hr of retrograde transport is shown in Table II. Approximately 25% of the grains were associated with lysosomes, which had a fractional volume of less than 5% and an LD of 5.8. Conventional MVBs were also labeled, and a large fraction of the grains (18%) were associated with elongated, membrane-limited profiles (EMPs) that could in some cases be identified as tubular bodies or elongated MVBs but were sometimes hard to distinguish from smooth endoplasmic reticulum (SER). Vesicles, including small "synaptic" and larger dense-cored vesicles, represented almost 20% of the fractional volume of the processes and were not preferentially labeled.

The distribution of label in the cell bodies after 24 hr of retrograde transport of ^{125}I-NGF is shown in Table III. Almost 60% of the label was associated with lysosomes, which had an LD of about 5. This is almost twice the lysosomal LD after five hr of retrograde transport. MVBs accounted for about 5% of the grains, with an LD of about 8. None of the other organelles, including the nucleus or nuclear membrane, was preferentially labeled.

The distribution of label in the proximal processes after 24 hr of retrograde transport of ^{125}I-NGF is shown in Table IV. The radioactivity was highly concentrated in lysosomes, which accounted for about 30% of the grains and had an LD of 13. MVBs and EMPs together accounted for about 8% of the label and were moderately labeled with an LD of approximately 2.

Thus, as retrograde transport proceeded, the label in the cell body tended to accumulate in the lysosomes; the percentage of the grains associated with lysosomes increased from about 45% to about 60% and the LD of the lysosomes increased from 2.7 to 5.2. The MVBs also tended to be more heavily labeled at 24 hr. Within the proximal neurites elongated profiles were associated with many more grains at five hr than at 24 hr, while at both time points the majority of the grains were associated with lysosomes.

Lysosomotropic Agents

The lysosomal blocking agents chloroquine (CQ) and methylamine (MA), in concentrations comparable to those used in previous studies [13,24], had profound effects on the morphology of lysosomes in sympathetic neurons in mass cultures. The appearance of cells that were exposed to ^{125}I-NGF for 22 hr in the presence of 0.05 mM CQ is shown in Figure 4. The lysosomes were enlarged and appeared engorged with membranous material. Other organelles such as mitochondria, the Golgi apparatus, and the rough endoplasmic reticulum appeared to be relatively unaffected. Under these conditions the cells effectively internalized ^{125}I-NGF and many of the grains were associated with lysosomes. Figure 5 illustrates neurons that were exposed to ^{125}I-NGF for 22 hr in the presence of 10 mM MA. Again, the lysosomes were engorged with membranous material while mitochondria, Golgi apparatus, and endoplasmic reticulum appeared relatively unaffected.

The MVBs were affected in a different way by CQ than by MA. In cells incubated in CQ, the number of MVBs in the cell bodies was approximately half that

TABLE II. Five Hr of Retrograde Transport: Distribution of ^{125}I-NGF in the Proximal Neurites

	Grains[a] (%)	Fractional area	Labeling density[b]
Lysosomes	25.2	4.4	5.8
MVB	0.8	0.5	1.7
EMP[c]	18.3	<0.5	>>5.0
Vesicles	0.4	18.4	0.02

[a]Total number of grains scored (fine grain development) = 135. Structures such as mitochondria, microtubules, neurofilaments or the plasma membrane were not preferentially labeled. They are not included here, so the percentages do not add up to 100%.
[b]Calculated as in Table I.
[c]EMP = elongated membrane-limited profiles.

TABLE III. Twenty-four Hr of Retrograde Transport: Distribution of ^{125}I-NGF in the Cell Body

	Grains[a] (%)	Fractional area	Labeling density[b]
Nucleus (total)	3.2	21.5	0.1
Nuclear membrane	0.5	2.0	0.2
Cytoplasmic organelles			
Lysosomes	57.9	11.2	5.2
MVB	4.8	0.6	8.3
Vesicles	0.1	1.1	0.1
Golgi	1.2	2.5	0.5
Mitochondria	8.7	10.6	0.8

[a]Total number of grains scored (fine grain development) = 3843. Structures such as microtubules, endoplasmic reticulum, or plasma membrane were not preferentially labeled. They are not included here, so the percentages do not add up to 100%.
[b]Calculated as in Table I.

TABLE IV. Twenty-four Hr of Retrograde Transport: Distribution of ^{125}I-NGF in the Proximal Neurites

	Grains[a] (%)	Fractional area	Labeling density[b]
Lysosomes	30.1	2.3	13.2
MVB	1.1	<0.5	>2.0
EMP[c]	6.7	3.6	1.8
Vesicles	1.3	20.5	0.06

[a]Total number of grains scored (fine grain development) = 445. Structures such as mitochondria, microtubules, neurofilaments, or the plasma membrane were not preferentially labeled. They are not included here, so the percentages do not add up to 100%.
[b]Calculated as in Table I.
[c]EMP = elongated membrane-limited profiles.

in control cells, or in cells incubated in MA. Following CQ treatment, the MVBs tended to be small and compact, while MVBs in MA-treated cells often appeared large and empty, with a clear lumen and few vesicles.

Preliminary measurements of ^{125}I-NGF associated with cells in similar cultures showed that in the presence of CQ or MA, the total amount of radioactivity associated with the cells was greater than in the controls. Specific association of NGF with the

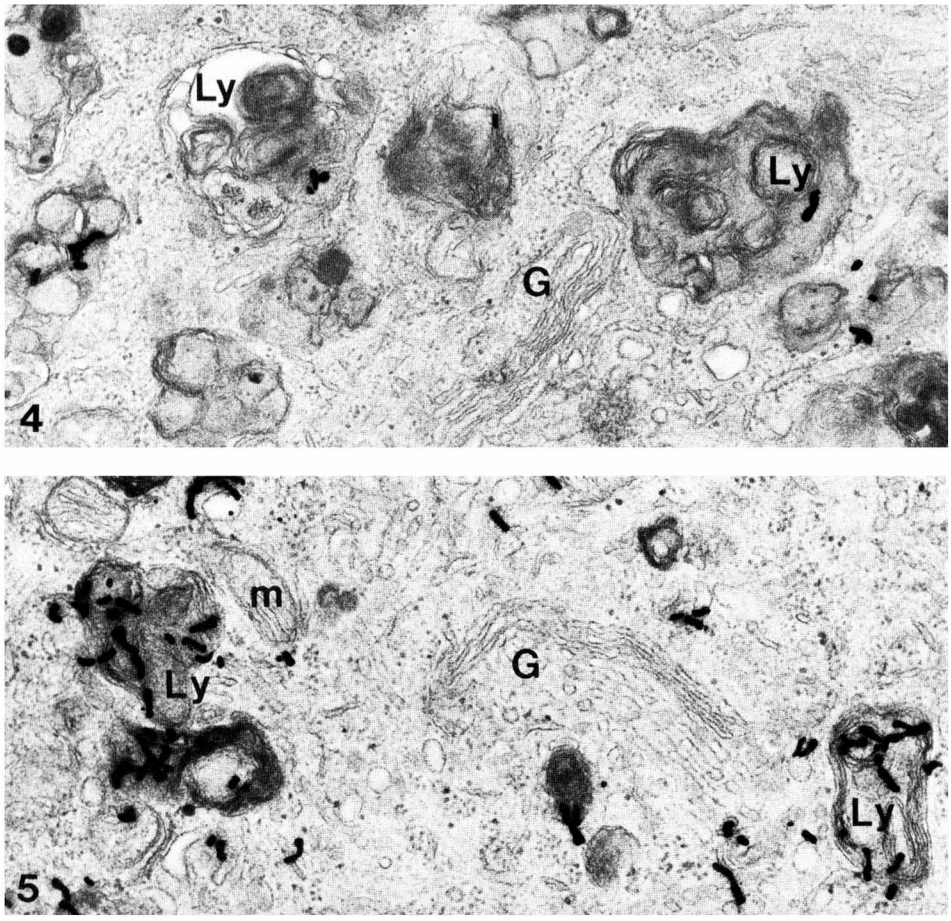


Fig. 4. Autoradiogram of a neuronal cell body that had been exposed to ^{125}I-NGF in the presence of 0.05 mM chloroquine for 22 hr. Note the swollen lysosomes (Ly). Fine grain development. G, Golgi apparatus. ×32,400.

Fig. 5. Autoradiogram of a neuronal cell body that had been exposed to ^{125}I-NGF in the presence of 10 mM methylamine. Fine grain development. Ly, lysosome; G, Golgi apparatus; m, mitochondrion. ×32,400.

cells did not seem to be greatly affected, but the amount of nonspecific association of label with the cells was increased.

The distribution of label in neuronal cell bodies following 22 hr of incubation in ^{125}I-NGF in the presence of 0.05 mM CQ is shown in Table V. The lysosomes accounted for almost two-thirds of the grains and the LD was rather high (about 6). MVBs accounted for very few of the grains and represented an extremely low fractional volume (0.04%). The smaller fractional volume of the MVBs was due to the fact that the MVBs were smaller and fewer in number in CQ-treated cells. Other organelles, including the nucleus and nuclear membrane, were unlabeled.

TABLE V. 0.05 mM Chloroquine for 22 Hr: Distribution of ^{125}I-NGF in the Cell Body

	Grains[a] (%)	Fractional area	Labeling density[b]
Nucleus	0	7.9	0
Nuclear membrane	0	0.2	0
Cytoplasmic organelles			
Lysosomes	64.3	10.4	6.2
MVB	0	0.04	0
Vesicles	0.5	1.4	0.4
Golgi	1.1	3.3	0.3
Mitochondria	3.2	7.3	0.4

[a]Total number of grains scored (Microdol X development) = 243. Structures such as microtubules, endoplasmic reticulum, or plasma membrane were not preferentially labeled. They are not included here, so the percentages do not add up to 100%.
[b]Calculated as in Table I.

TABLE VI. 10 mM Methylamine for 22 Hr: Distribution of ^{125}I-NGF in the Cell Body

	Grains[a] (%)	Fractional area	Labeling density[b]
Nucleus	2.4	12.2	0.2
Nuclear membrane	2.0	1.0	2.0
Cytoplasmic organelles			
Lysosomes	28.0	16.6	1.7
MVB	3.6	3.6	1.0
Vesicles	1.2	2.6	0.4
Golgi	5.3	5.8	0.9
Mitochondria	10.6	4.4	2.4

[a]Total number of grains scored (Microdol X development) = 665. Structures such as microtubules, endoplasmic reticulum, or plasma membrane were not preferentially labeled. They are not included here, so the percentages do not add up to 100%.
[b]Calculated as in Table I.

The distribution of grains following incubation of cells with ^{125}I-NGF for 22 hr in the presence of 10 mM MA is shown in Table VI. Mature lysosomes accounted for almost 30% of the grains, which is within the normal range, and MVBs accounted for 3.6% of the grains, which is lower than usual. On the other hand, the fractional volume of the MVBs was quite a bit larger than usual, 3.6% rather than less than 1%. Both lysosomes and MVBs had low LDs (1.7 and 1.0, respectively). Structures usually not labeled above background, such as mitochondria and ground cytoplasm, accounted for more than usual of the grains. The nuclear membrane was in contact with 2% of the grains and had an LD of 2, while mitochondria had an LD of 2.4.

We conclude that the lysosomotropic agents do not inhibit the uptake of ^{125}I-NGF into sympathetic neurons, but that they do result in more nonspecific association of the label with the cells and in a different pattern of localization within the cell. Neither CQ nor MA caused a significant accumulation of ^{125}I-NGF in the nucleus.

DISCUSSION

In this study we used electron microscopic autoradiography to investigate the fate of ^{125}I-NGF as it interacts with sympathetic neurons in culture. In the neurites, ^{125}I-NGF appeared to be transported retrogradely within lysosomes, multivesicular bodies, and, especially at the earlier time point, within elongated membrane-limited profiles. Once in the cell body, the ^{125}I-NGF was associated primarily with lysosomes and MVBs, organelles that have been shown in other situations to contain degradative enzymes [25]. We found no evidence for the accumulation of NGF in the nucleus. In view of the presence of internalized NGF within lysosomes, we also investigated the effects of the lysosomal inhibitors chloroquine and methylamine on lysosomal organelles and on the localization of ^{125}I-NGF within the cell following internalization.

Retrograde Transport

In examining the cell bodies of neurons that had retrogradely transported ^{125}I-NGF for 5 or 24 hr, we found that the largest percentage of grains was associated with conventional lysosomes. This is consistent with the fact that as retrograde transport proceeds, the concentration of NGF in the cell bodies eventually reaches a steady state, with degradation products appearing in the incubation medium (unpublished observations). It is also consistent with in vivo studies of retrograde transport of NGF by sympathetic neurons using a horseradish peroxidase (HRP) conjugate of NGF [17] or ^{125}I-NGF [9,10].

In the proximal neurites, conventional lysosomes and MVBs were associated with the largest percentage of grains. This is in agreement with other studies showing that the majority of organelles accumulating distal to an obstruction of axonal transport were lysosomes or residual bodies [26,27]. It may be significant, however, that at 5 hr, in early stages of axonal transport, the organelles with the highest LD were a variety of elongated membranous profiles (EMPs) which in our analysis probably included some SER as well as tubular bodies and elongated MVBs. These organelles may be analogous to the SER-like organelles reported to be involved in the retrograde transport of HRP-conjugated NGF in vivo [10,17]. In serially sectioned axons that were retrogradely transporting HRP in the chick optic nerve, it was determined that the SER was definitely not involved in the transport of HRP, but that there were other elongated membrane-limited profiles that did contain HRP [28]. Some of these profiles were identified as MVBs and others as tubular bodies. It is possible that both NGF and HRP are transported in organelles that are distinct from the SER. On the other hand, a different population of organelles may be responsible for the transport of NGF, which is internalized by a specific uptake mechanism, than for the transport of substances internalized by fluid endocytosis, such as HRP.

Localization of NGF Within Lysosomes

The localization of intact ^{125}I-NGF in lysosomes [3,9,10] raises questions about the functional significance of the presence of NGF in what are considered to be degradative organelles. There are a number of possible explanations for the lysosomal involvement: (a) the NGF in the lysosomes has already had its effect, perhaps through the intermediary of an as yet unidentified second messenger, and the NGF is simply being cleared from the cell; (b) the NGF has had its effect, but the lysosomal step is

necessary in order for membrane receptors to be recycled; (c) there is a subset of lysosomes that do not contain active degradative enzymes but that are specialized as storage sites for NGF; and (d), the lysosome plays a necessary role in mediating the response to NGF. An example of the last process is seen in the case of diphtheria toxin, where lysosomal processing permits the toxin to penetrate the membrane of the lysosome and gain access to the cytoplasm of the cell [29,30]. The low intralysosomal pH appears to be a critical factor [29,30]. In the case of epidermal growth factor (EGF), the degradation of internalized EGF can be blocked with protease inhibitors without blocking mitogenesis, a long-term biological effect [31], while agents that affect intralysosomal pH do block EGF's mitogenic effect [32].

Lysosomotropic Agents

Since lysosomal organelles may be important in the processing of NGF by its target cells, we were interested in the consequences of disrupting lysosomal function. The agents we used are thought to work by raising intralysosomal pH [33]. Superficially, both CQ and MA appeared to have the same effects on the cells; the lysosomes became engorged with membranous material, perhaps as a consequence of the inhibition of degradative activity. In most other respects the cells appeared relatively normal. There were some differences in the morphological effects of the two agents, however; in CQ the number of MVBs was reduced and those that were present were fairly compact, while in MA the number of MVBs was about normal but many were larger, with an empty, vacuolated appearance. In CQ the lysosomes were highly labeled, so the cells were not only internalizing NGF but they were also translocating it into lysosomes as usual.

Under the conditions of our experiments there was little evidence for nuclear labeling in either CQ or MA, except for a suggestion of label associated with the nuclear envelope in MA. (It should be pointed out that in MA there were more grains than usual associated with cytoplasmic structures such as mitochondria, ribosomes, and microtubules, as though some ^{125}I-NGF had leaked into the cytoplasm. We cannot rule out some damage to the cells.) In the case of CQ, we saw no nuclear labeling at all. It has been reported that under similar incubation conditions, PC-12 cells treated with CQ exhibit high levels of nuclear labeling [13], but morphological verification of the nuclear labeling has not been done. It is of course possible that sympathetic neurons and PC-12 cells process NGF in different ways.

Intracellular Pathway

It would be useful to know what steps occur and what organelles are involved between the initial binding of NGF to plasma membrane receptors and its eventual degradation in lysosomal organelles. By using a ferritin conjugate of EGF, it has been possible to trace the intracellular pathway of EGF in A-431 cells at the ultrastructural level [34,35]. EGF appears to be taken up initially in small endocytic vesicles that rapidly fuse with MVBs. The MVBs themselves fuse with lysosomes and become a form of secondary lysosome, and within an hour much of the EGF is degraded [35]. Since we found that in sympathetic neurons exposed to ^{125}I-NGF, MVBs are the most highly labeled organelles, it seems plausible that a similar sequence of events might occur in the case of NGF. There seem to be some differences, however, between the behavior of EGF and NGF. In the case of EGF [35], lysosomal inhibitors tended to

inhibit the progression from MVBs to lysosomes; we found that in the presence of CQ lysosomes were more highly labeled than usual, and that MVBs were fewer and less heavily labeled. Our results could be interpreted to mean that the CQ actually increased the progression from MVBs to lysosome. Another difference between the two systems is that NGF is not degraded as rapidly as EGF is [4,5].

It has been suggested that NGF in PC-12 cells acts either in the cytoplasm [14] or by binding to the nuclear envelope [13]. It would seem unlikely that the NGF acts by binding to cytoplasmic structures, since unless the membranes of NGF-containing organelles are frankly ruptured, or a specific mechanism allows NGF to penetrate the membrane, it would be difficult for NGF located inside the organelles to get into the cytoplasm. In any case, direct injection of NGF into the cytoplasm of NGF sensitive PC-12 cells proved to be ineffective in promoting neuritic outgrowth, whether it was injected by microelectrode [36] or by means of fusion with NGF-filled erythrocyte ghosts [37]. In addition, a recent ultrastructural autoradiographic study has failed to demonstrate the concentration of internalized ^{125}I-NGF in the nuclei of healthy PC-12 cells [38]. Thus the function of internalized NGF remains to be established.

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