Rapid Retrograde Tyrosine Phosphorylation of trkA and Other Proteins in Rat Sympathetic Neurons in Compartmented Cultures

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Abstract. According to the current theory of retrograde signaling, NGF binds to receptors on the axon terminals and is internalized by receptor-mediated endocytosis. Vesicles with NGF in their lumina, activating receptors in their membranes, travel to the cell bodies and initiate signaling cascades that reach the nucleus. This theory predicts that the retrograde appearance of activated signaling molecules in the cell bodies should coincide with the retrograde appearance of the NGF that initiated the signals. However, we observed that NGF applied locally to distal axons of rat sympathetic neurons in compartmented cultures produced increased tyrosine phosphorylation of trkA in cell bodies/proximal axons within 1 min. Other proximal proteins, including several apparently localized in cell bodies, displayed increased tyrosine phosphorylation within 5–15 min. However, no detectable ^{125}I-NGF appeared in the cell bodies/proximal axons within 30–60 min of its addition to distal axons. Even if a small, undetectable fraction of transported ^{125}I-NGF was internalized and loaded onto the retrograde transport system immediately after NGF application, at least 3–6 min would be required for the NGF that binds to receptors on distal axons just outside the barrier to be transported to the proximal axons just inside the barrier. Moreover, it is unlikely that the tiny fraction of distal axon trk receptors located near the barrier alone could produce a measurable retrograde trk phosphorylation even if enough time was allowed for internalization and transport of these receptors. Thus, our results provide strong evidence that NGF-induced retrograde signals precede the arrival of endocytotic vesicles containing the NGF that induced them. We further suggest that at least some components of the retrograde signal are carried by a propagation mechanism.

NGF, the best characterized neurotrophin, elicits differentiation, survival, and neurite growth in sympathetic neurons. Many NGF effects are mediated by the binding of NGF to the receptor tyrosine kinase (trk)[1], trkA (Loeb et al., 1991; Loeb and Greene, 1993; Ibáñez et al., 1992), which induces rapid tyrosine autophosphorylation of trk and subsequent tyrosine phosphorylations of several second messenger proteins (Kaplan et al., 1991*a*,*b*; Klein et al., 1991; Jing et al., 1992). Activation of these proteins by tyrosine phosphorylation is believed to play an important role in mediating the biological responses of neurons to NGF.

Evidence indicates that the immediate neurite growth–promoting action of NGF involves mechanisms at or near the site of NGF binding to the axon terminals (Campenot 1977, 1982, 1987; Campenot et al., 1994). This suggests that trk phosphorylation leads to the activation of second messenger systems in the growth cones that directly couple to local growth mechanisms. In contrast, biological effects of NGF such as promotion of cell survival (Levi-Montalcini, 1976, 1987) and changes in gene expression (Mathew and Miller, 1990; Miller et al., 1991; Ma et al., 1992; Wyatt and Davies, 1995; Toma et al., 1997) involve retrograde signals that travel from the axon terminals to the cell body and nucleus.

NGF is retrogradely transported along axons of sympathetic neurons and neural crest–derived sensory neurons (Hendry et al., 1974*a*; Stöckel et al., 1975; Claude et al., 1982; Korsching and Thoenen, 1983; Palmatier et al., 1984). These observations support a favorite model for retrograde signaling: NGF binds to and activates trk receptors on the axon terminals and is internalized by receptor-mediated endocytosis. Then, the endocytotic vesicles carrying trk in their membranes, activated by NGF in their lumina, are retrogradely transported to the cell body. Once in the cell body, activated trk phosphorylates second

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1. *Abbreviation used in this paper*: trk, receptor tyrosine kinase.

messenger proteins that transmit signals to the nucleus, resulting in altered gene expression (for reviews see Korshing, 1993; Campenot, 1994). Recent evidence that phosphorylated trk is transported in the axonal retrograde transport system supports this theory of retrograde signaling by NGF (Ehlers et al., 1995; Grimes et al., 1996).

Discovering the mechanisms of retrograde signaling in NGF-responsive neurons is of vital importance, serving as a model for many other types of neurons and trophic factors. This information is indispensable for understanding neural development and will help in efforts to devise ways to promote neuronal survival and repair after disease or injury. The compartmented culture model is an ideal means to investigate the mechanisms of retrograde signaling. In compartmented cultures, the cell bodies and proximal neurites reside in center compartments, while distal neurites extend into left and right distal compartments. We used these cultures to apply NGF locally to distal neurites and to observe the appearance of NGF-induced tyrosine phosphorylations and the arrival of ^{125}I-NGF in the cell bodies/proximal neurites. Our results indicate that NGF binding to distal neurites induces the tyrosine phosphorylation of trk and other proteins in the cell bodies/proximal neurites long before the NGF is internalized and delivered by retrograde transport. While our results do not rule out the possibility that some NGF-induced retrograde signals could be carried by retrograde NGF transport, retrograde transport cannot be the only mechanism. Rather, our results suggest that at least some retrograde NGF signals are carried by a propagation mechanism.

Materials and Methods

Culture Procedures

Superior cervical ganglia were dissected from newborn rats (Sprague-Dawleys supplied by the University of Alberta Farm, Alberta, Canada) as previously described (Campenot et al., 1991), subjected to trypsin and mechanical dissociation, and plated into collagen-coated culture dishes. For mass cultures neurons were plated into 24-well Linbro tissue culture dishes (ICN Biomedicals, Inc., Aurora, OH) at a density of one ganglion per well. Neurons were plated into compartmented cultures as previously described (Campenot et al., 1994). For most experiments compartmented cultures were maintained for 2 wk after plating with NGF supplied in all compartments at 10 ng/ml. Fig. 1 shows a single track from a culture raised under these conditions and retrogradely labeled overnight with the lipophilic, fluorescent dye, FM-145 (Molecular Probes, Eugene, OR).

Culture Media

L15 medium without antibiotics (Gibco Laboratories, Grand Island, NY) was supplemented with additives prescribed by Hawrot and Patterson (1979) including bicarbonate and methylcellulose. Rat serum (2.5%, provided by the University of Alberta Laboratory Animal Services) and ascorbic acid (1 mg/ml) were supplied in medium given to mass neuronal cultures and medium given to the center compartments of the compartmented cultures containing the cell bodies. Culture medium was routinely changed every 3–7 d. Nonneuronal cells were virtually eliminated by supplying 10 mM cytosine arabinoside in the mass neuronal cultures and center compartments of compartmented cultures during the first 6 d. Mass cultures were grown in medium containing 200 ng/ml NGF for 10–14 d before experimental treatment. Compartmented cultures were grown in 10 ng/ml NGF in all three compartments for 10–14 d.

Experimental Treatments

The experimental variable in these experiments consisted of various concentrations of NGF, K-252a, and anti-NGF. NGF (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) stock was 20 mg/ml in PBS. The standard NGF concentration used in cultures ranged from 10–200 ng/ml. K-252a (Kamiya Biomedical Co., Thousand Oaks, CA) was prepared as a 2 mM stock in DMSO and stored at 4°C. The 2 mM stock was diluted to 500 nM in culture medium. The DMSO concentration with 500 nM K-252a was 0.025%. Anti-NGF affinity-purified sheep IgG (Cedarlane Laboratories Ltd.) was used at a final concentration of 24 nM.

Protein Tyrosine Phosphorylation

After experimental treatment, cultures were washed twice with ice-cold TBS, and cell extracts from both the cell body/proximal axon compartments and the distal axon compartments were collected separately into sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM 2-mercaptoethanol, 0.001% bromophenol blue). Extracts were run on 8% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose (Hyperbond; Amersham, Oakville, Ontario, Canada) using a semidry transfer unit (Hoefer Pharmacia Biotech Inc., San Francisco, CA) and immunoblotted using anti-phosphotyrosine 4G10 antibody (Upstate Biotechnology Inc., Lake Placid, NY). Immunoreactivity was determined using enhanced chemiluminescence (ECL; Amersham). Data were quantified using an Ultroscan XL laser densitometer (Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

Immunoprecipitation

After treatment, cultures were washed with ice-cold TBS and solubilized in lysis buffer (10 mM Tris-HCl, pH 7.4, 1% NP-40, 10% glycerol, 1 mM PMSF, 1 mM sodium orthovanadate, 5 μg/ml leupeptin, 5 μg/ml aprotinin). Extracts from mass neuronal cultures were pooled in microcentrifuge tubes, homogenized using a microhomogenizer pestle (Mandel Scientific Co. Ltd., Guelph, Ontario, Canada), and centrifuged in an Eppendorf microcentrifuge (Eppendorf North America Inc., Madison, WI) for 30 s to remove cell debris. Extracts were then normalized for total protein by bicinchoninic acid protein determination kit (Sigma Chemical Co., St. Louis, MO) and immunoprecipitated with a polyclonal antibody against anti-trk 203B (provided by David Kaplan, Montreal Neurological Institute, Montreal, Canada). Immunoprecipitates were then run on 8% SDS-polyacrylamide gels, transferred to nitrocellulose, immunoblotted using anti-phosphotyrosine 4G10 antibody, and detected using ECL as described above. Immunoprecipitations for trk in compartmented cultures were performed on extracts from the cell body/proximal axon compartment of 14–18 cultures. Data were quantified using an LKB Ultroscan XL laser densitometer.

Equalization of Sample Loading

Attempts to detect trk by Western blotting with anti-trk 203B antibody were unsuccessful, presumably because compartmented cultures, which contain ~1,500 neurons per dish, do not provide sufficient trk protein for detection. Therefore, it was not feasible for us to reprobe our anti-phosphotyrosine blots to verify equal amounts of trk between control and experimental groups. Therefore, to equalize sample loading, we always used an equal number of sister cultures for control and experimental groups that had been treated identically from the initial day of plating. We have evidence that using equal culture numbers is effective since our previous observations showed that activation of trk by global application of 200 ng/ml NGF followed by immunoprecipitation with anti-trk and immunoblotting with anti-phosphotyrosine antibodies reproducibly detected equal amounts of trk protein under a variety of experimental conditions (Toma et al., 1997). In the present study this was confirmed in four replicate experiments in which equal aliquots of extracts from control and NGF treatment groups taken before trk immunoprecipitation were immunoblotted with anti–α-tubulin and anti-erk antibodies. Immunoblots showed that control and experimental groups had equal amounts of tubulin and erk, while retrograde tyrosine phosphorylation of trk was increased after 1 min of NGF exposure at the distal axons. Moreover, the NGF-induced retrograde tyrosine phosphoryation was highly repeatable, replicated seven times for the retrograde increase in trk phosphorylation

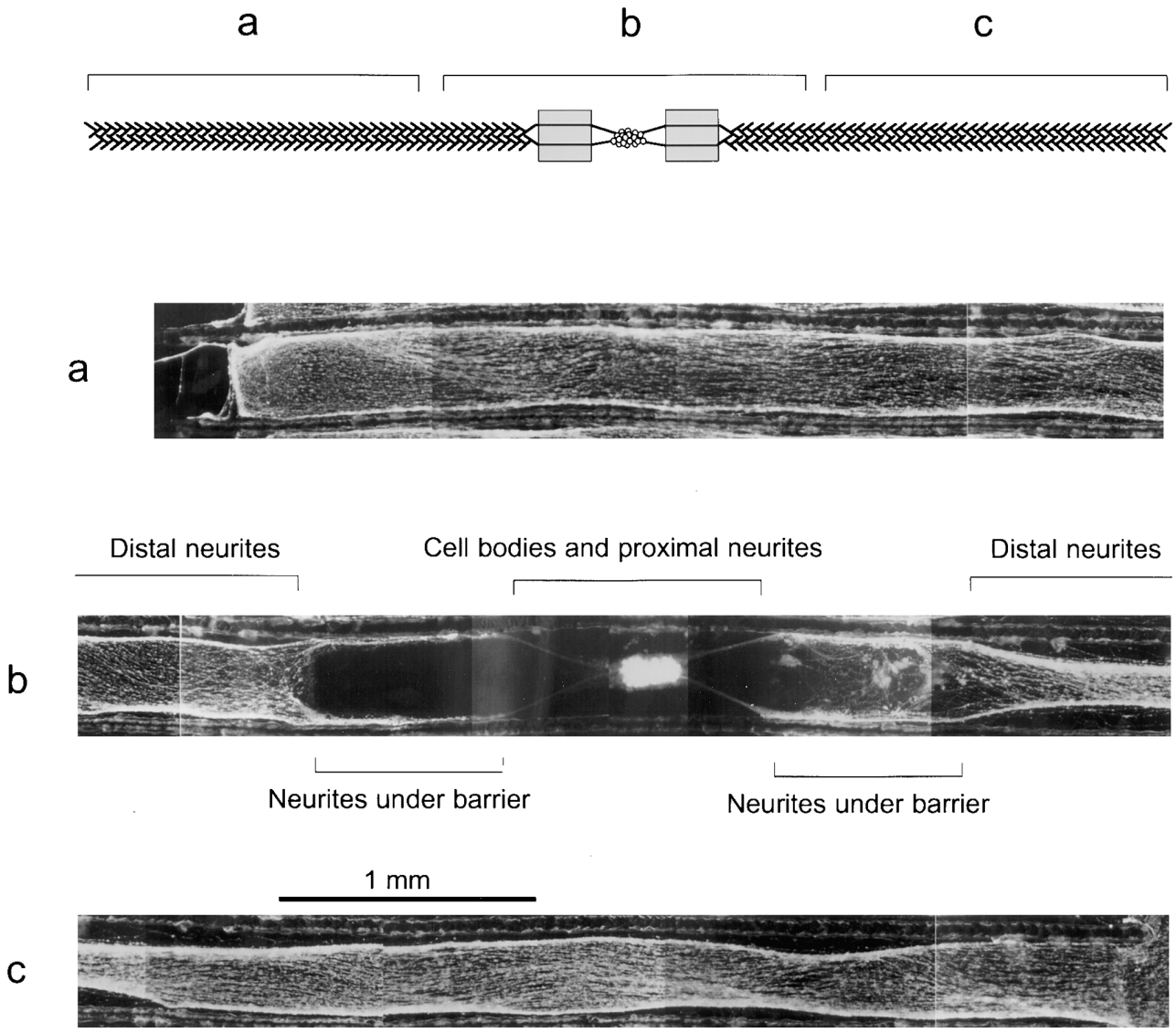

Figure 1. Sympathetic neurons in compartmented cultures. Photomicrographs show a single track in a compartmented culture of sympathetic neurons raised for 14 d with 10 ng/ml NGF in all compartments. The culture was labeled overnight with the lipophilic fluorescent dye, FM-145 (4 μM), added to the left and right compartments. Labeling of cell bodies and proximal axons occurred by retrograde transport from labeled distal axons. The letters on photomicrographs are keyed to the schematic diagram. *b* shows a cluster of cell bodies in the center compartment. Fine bundles of proximal axons connect the cell bodies to the neurites under the barrier that tend to hug the scratches. Upon emerging into the distal compartments, the neurites spread out to cover the collagen track and extend many millimeters into the left (*a*) and right (*c*) compartments.

at 1 min and 12 times for the retrograde increase in p140 phosphorylation at 10 min after distal NGF application.

Immunoblotting of Tubulin and erk

In some experiments after treatment, aliquots of cell extracts were collected and analyzed on immunoblots using either 1 μg/ml monoclonal α-tubulin (clone DM 1A; Sigma Chemical Co.) or 0.1 μg/ml polyclonal erk antibody (691; Santa Cruz Biotechnology Inc., Santa Cruz, CA). The erk antibody detects both p44 and p42 erks. Except for the use of these antibodies, the procedure was the same as previously described above for protein tyrosine phosphorylation.

Radioiodination of NGF and Retrograde Transport Assay

Radioiodination of NGF and retrograde transport assays were performed as previously described by Ure and Campenot (1997). All transport assays were performed on compartmented cultures of sympathetic neurons grown for 10–14 d in 10 ng/ml NGF in all compartments. ^{125}I-NGF was applied at a concentration of 40×10^6 cpm/ml (200 ng/ml) to distal axon compartments for times ranging from 1 min to 24 h. Radioactivity present in both the center compartment medium and the cell bodies/proximal axon extracts was quantified using a 1470 gamma counter (Wallac, Gaithersburg, MD).

Results

Concentration Dependence and Time Course of trk Activation by NGF in Mass Cultures

Before attempting to investigate retrograde tyrosine phosphorylations in compartmented cultures, the NGF-induced

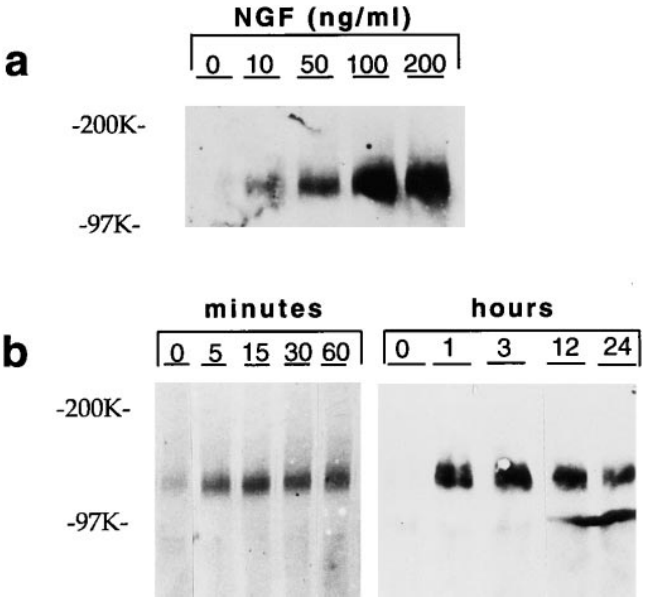

Figure 2. NGF-dependent tyrosine phosphorylation of trk in mass cultures. Cultures of rat sympathetic neurons grown in 200 ng/ml NGF were given NGF-free media for 2–4 h before experimental treatment. In each experiment, samples containing equal amounts of protein were immunoprecipitated using anti-trk antibody and immunoblotted using anti-phosphotyrosine antibody. Molecular mass markers in kD are indicated on the left of each blot. (*a*) Dose–response of trk tyrosine phosphorylation. Cultures were given varying concentrations of NGF (0–200 ng/ml) for 10 min. (*b*) Time course of trk tyrosine phosphorylation. Cultures were given 200 ng/ml NGF for times ranging from 0–24 h.

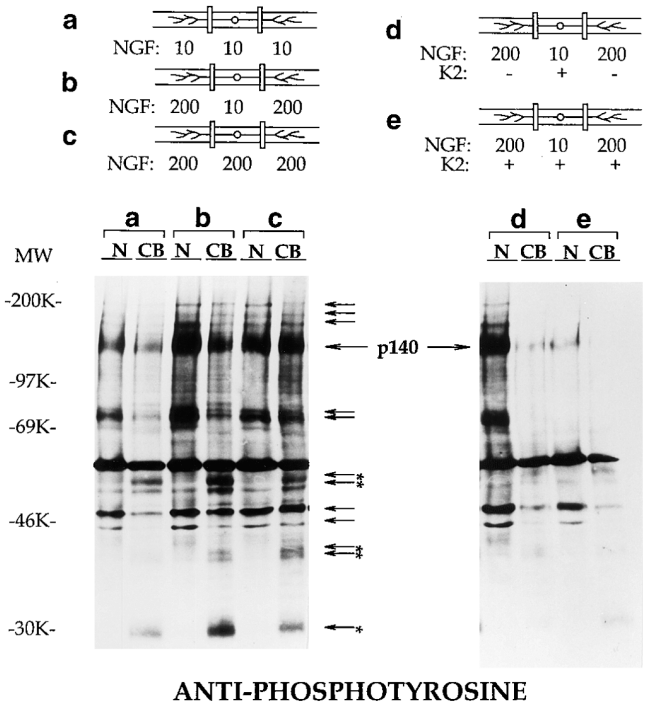

Figure 3. Protein tyrosine phosphorylation in response to different distributions of NGF. Compartmented cultures of rat sympathetic neurons were grown for 2 wk in 10 ng/ml NGF in all compartments. Cultures were treated for 10 min with either: (*a*) 10 ng/ml NGF applied to all compartments; (*b*, *d*, and *e*) 10 ng/ml NGF applied to cell bodies/proximal axons and 200 ng/ml NGF applied to distal neurites; or (*c*) 200 ng/ml NGF applied to all compartments. In center compartments (*d*) and in all compartments (*e*), 500 nM K-252a (*K2*) was supplied, starting 30 min before the NGF treatments. Cell extracts were collected from the cell body/proximal neurite compartments (*CB*) and the distal neurite compartments (*N*). To ensure comparability between treatments, all cultures used were sister cultures, and each group contained the extracts pooled from three cultures. The extracts were analyzed by immunoblotting with anti-phosphotyrosine (4G10) antibody. (*Arrows*) Tyrosine phosphorylation of proteins produced by distally applied NGF. (*Asterisks*) Tyrosine-phosphorylated proteins found only in cell bodies/proximal axons. The position of trk migration is indicated. Molecular mass markers are indicated on the left.

trk phosphorylation was characterized in mass cultures of rat sympathetic neurons. Cultures initially grown for 2 wk in medium supplied with 200 ng/ml NGF were given NGF-free media for 2–4 h, and then given medium containing NGF at concentrations ranging from 10–200 ng/ml. Cell extracts were collected, and samples containing equal amounts of protein were immunoprecipitated with anti-trk antibody and analyzed for tyrosine phosphorylation by immunoblotting with anti-phosphotyrosine antibody (see Materials and Methods). Tyrosine phosphorylation of trk increased substantially with concentrations of NGF ranging from 10–100 ng/ml (Fig. 2 *a*). This contrasts with PC12 cells that show maximal trk autophosphorylation at 10 ng/ml NGF (Kaplan et al., 1991*b*). We used 200 ng/ml NGF in all experiments to ensure maximal activation of trk.

To assess the time course of NGF-induced trk phosphorylation, cultures grown in 200 ng/ml NGF were given NGF-free medium for 2 h and then given 200 ng/ml NGF for various times. trk tyrosine phosphorylation was detected within 5 min and persisted for at least 24 h (Fig. 2 *b*), consistent with a role for trk autophosphorylation in mediating long-term, not just transient, signals.

Local and Retrograde NGF-induced Protein Tyrosine Phosphorylations

To analyze the tyrosine phosphorylations that occur both locally and retrograde to the binding of NGF, we used three-compartmented cultures. Neurons were plated in center compartments, and their axons extended under silicone grease barriers and entered into separate distal axon compartments (Campenot, 1992). Using this system, distal axons could be locally exposed to increased NGF, and protein tyrosine phosphorylation could be measured in cell extracts separately obtained from distal axons and from cell bodies/proximal axons. Initially, neurons were grown in 10 ng/ml NGF in all compartments for 2 wk (Fig. 1). Then 200 ng/ml NGF was given either only in the distal compartments or in all compartments for 10 min. Control cultures received the same changes of medium, but with NGF maintained at 10 ng/ml in all compartments. Each group consisted of three cultures, extracts of which were analyzed by anti-phosphotyrosine immunoblotting. 10-min exposure to distal NGF produced tyrosine phosphorylations locally in the distal neurites as well as retrograde phosphorylations of proteins in the cell bodies/proximal

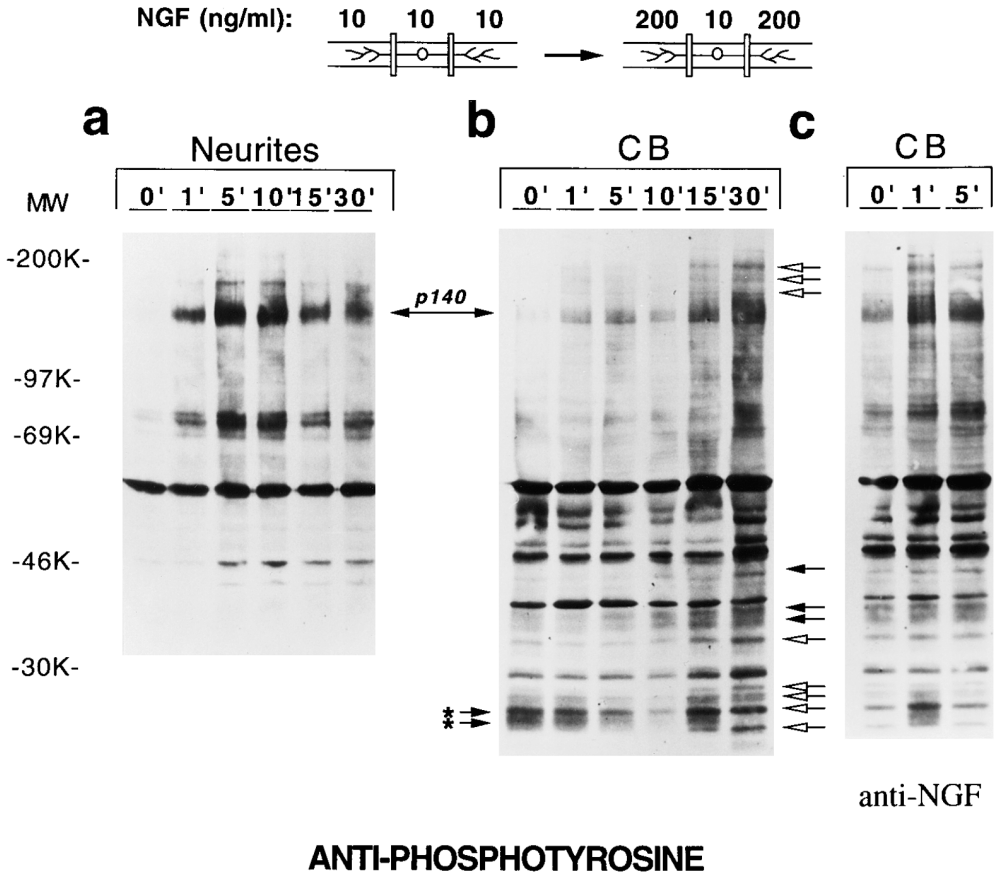

Figure 4. Time course of local and retrograde NGF-induced tyrosine phosphorylations. Compartmented cultures of rat sympathetic neurons grown for 2 wk in 10 ng/ml NGF in all compartments were supplied with 200 ng/ml NGF in distal compartments for the times indicated. All cultures were sister cultures. Cell extracts were collected directly into sample buffer and analyzed by immunoblotting with anti-phosphotyrosine antibody. Molecular mass markers in kD for all gels are indicated on the left. Increasing tyrosine phosphorylation of proteins is indicated by arrows (filled arrows are proteins appearing within 10 min and open arrows are proteins appearing by 15 min). (*Asterisks*) Dephosphorylation of proteins. The position of trk is indicated. (*a*) Results from extracts of distal neurites of three cultures for each time point. (*b*) Results from cell bodies and proximal neurites (*CB*) of five cultures for each time point. The neurites in *a* were from a subgroup of the cultures used in *b*. (*c*) Results from cell bodies and proximal neurites of five cultures for each time point that were given 24 nM anti-NGF antibody to the center compartment 10 min before distal application of 200 ng/ml NGF.

neurites not directly exposed to increased NGF (Fig. 3 *b*). Proteins displaying a retrograde tyrosine phosphorylation included a band at 140 kD, the apparent molecular mass of trk, and several other proteins with apparent molecular masses ranging from 30–190 kD (Fig. 3, *arrows*). The pattern of tyrosine-phosphorylated proteins in the cell bodies/proximal axons was similar whether NGF was given only to the distal axons or globally to the entire surface of the neurons (Fig. 3 *c*). The cell bodies/proximal axons displayed five tyrosine-phosphorylated bands that were not present in the distal axons (apparent molecular masses 30, 36, 38, 50, and 55 kD; *asterisks*). Their relative absence from the distal compartments containing axons alone suggests that these phosphorylated proteins are localized to the neuronal cell bodies.

This experiment was repeated several times and the retrograde tyrosine phosphorylation of p140 and of the cell body–localized bands, p38, p36, and p30, was quantified by densitometry (see Fig. 6). Application of 200 ng/ml NGF to distal axons for 10 min produced a 3–4.5-fold increase in the tyrosine phosphorylation density of these proteins.

To determine to what extent the phosphorylation and activation of trk was involved in the retrograde tyrosine phosphorylation of these proteins, trk autophosphorylation was blocked by application of K-252a (Berg et al., 1992; Ohmichi et al., 1992; Tapley et al., 1992). Two groups of cultures given 200 ng/ml NGF in distal compartments were also treated with 500 nM K-252a either only in the center compartments containing cell bodies and proximal axons (Fig. 3 *d*) or in all compartments (Fig. 3 *e*). K-252a was given 30 min before distal application of NGF. When present in all compartments, K-252a blocked the NGF-induced tyrosine phosphorylations of all proteins. K-252a given only to the cell bodies/proximal axons selectively blocked retrograde phosphorylations without any apparent effect on the NGF-induced tyrosine phosphorylations in the distal axons.

Since we observed retrograde tyrosine phosphorylations 10 min after exposure of distal axons to NGF, we conducted an experiment to define the time course of tyrosine phosphorylations from 1 min (the shortest time practical in our system) to 30 min after NGF was given to distal axons. Neurons grown for 2 wk with 10 ng/ml NGF in all compartments were supplied with either 10 or 200 ng/ml NGF on their distal axons. Local tyrosine phosphorylation of the 140-kD protein in distal axons occurred within 1 min of distal NGF treatment, reaching a maximum by 5 min, and was maintained for 30 min (Fig. 4 *a*). Phosphorylations of proteins at 65, 70, 85, and 180 kD in distal axons also occurred within 1 min of distal NGF application. Tyrosine phosphorylation of two additional proteins at 42 and 44 kD occurred within 5 min. Tyrosine phosphorylation of all proteins observed in the distal axons was maintained for 30 min.

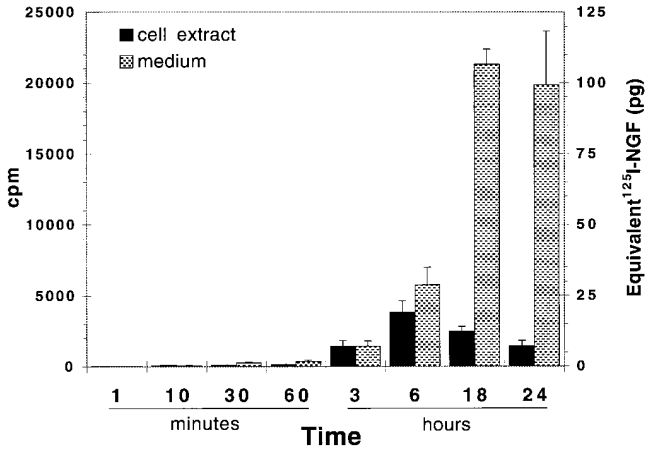

Figure 5. Time course of radiolabel accumulation in center media and cell extracts after addition of ^{125}I-NGF to distal compartments. Compartmented cultures of rat sympathetic neurons grown for 10–14 d in the presence of 10 ng/ml NGF where given 200 ng/ml ^{125}I-NGF to distal compartments for times ranging from 1 min to 24 h. All cultures were sister cultures. After the addition of ^{125}I-NGF to distal compartments, medium (*stippled bars*) and cell extracts (*filled bars*) from the center compartment were collected and assayed separately for radiolabel content for each individual culture (*cpm*; primary y-axis). Equivalent ^{125}I-NGF concentration (*pg*) was calculated and indicated on the secondary y-axis. Bars represent means (± SEM). The number of cultures for each time point was 7–10 except for 18 h, which was three cultures.

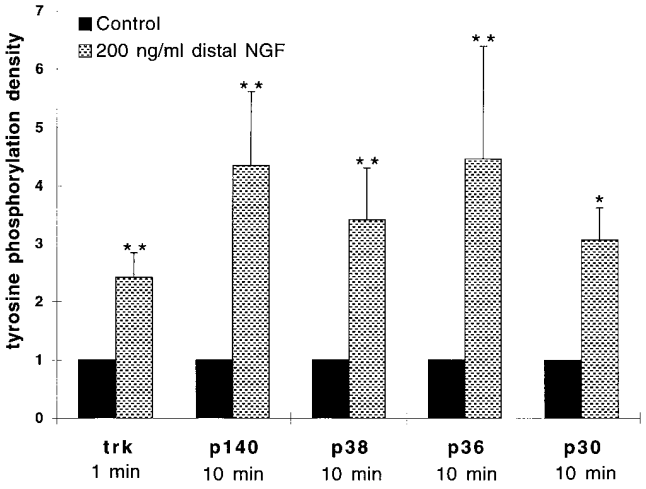

Figure 6. Quantitative analysis of retrograde phosphorylated proteins. Retrograde tyrosine phosphorylation occurring in the cell bodies/proximal axons of control neurons and neurons with 200 ng/ml NGF supplied to their distal axons was quantified by densitometry. The results are expressed relative to the values of the control response that was set as 1.0. (*Stippled bars*) Means of the NGF-treated groups; (*solid bars*) control levels. The proteins and time of NGF exposure are indicated on the x-axis. The sample sizes were 6, 6, 3, 3, and 4, respectively, and the error bars are ± SEM. The results of one experiment were not included in the analysis of p30 because of a low control density that gave an outlying NGF-induced increase of 62-fold. The statistical significance between NGF-treated and control neurons for each protein was tested by the paired sample *t* test and is indicated for each protein as the probability value (*P*). $P < 0.01$ (*double asterisks*) and $P < 0.05$ (*single asterisk*) relative to control neurons.

Retrograde tyrosine phosphorylation of the 140-kD protein appeared within 1 min of application of NGF to the distal axons followed by several other proteins within 10 (Fig. 4 *b*, *filled arrows*) and 15 min (*open arrows*) with increasing phosphorylation throughout the 30 min of observation. In addition to phosphorylation, dephosphorylation of some proteins occurred within 5–10 min (*asterisks*). Similar results were obtained in experiments in which 24 nM of anti-NGF antibody was present in the center compartments to ensure that the retrograde phosphorylation did not result from direct exposure to extracellular NGF (Fig. 4 *c*).

Retrograde Transport of ^{125}I-NGF

To determine the relationship between the appearance of retrograde tyrosine phosphorylations and the appearance of retrogradely transported NGF, the retrograde transport of ^{125}I-NGF was measured in experiments similar to the retrograde tyrosine phosphorylation experiments. Compartmented cultures were grown with 10 ng/ml NGF in all compartments for 10–14 d, and then given 200 ng/ml ^{125}I-NGF in distal compartments for times ranging from 1 min to 24 h. Extracts of cell bodies/proximal axons from the center compartments and the medium bathing them were collected and assayed separately. Previous results have shown that the ^{125}I accumulated in the cell bodies/proximal axons represents intact NGF, and the ^{125}I in the medium represents low molecular weight breakdown products released into the medium after breakdown of transported NGF (Ure and Campenot, 1994, 1997). We observed no retrogradely transported ^{125}I-NGF within 10 min, and little, if any, during the first hour (Fig. 5). After 1 h the retrograde transport of ^{125}I-NGF greatly increased. The transport was specific since a 100-fold excess of unlabeled NGF reduced the accumulation of ^{125}I-NGF in cell bodies and proximal axons at 24 h by 95%.

Rapid Retrograde Tyrosine Phosphorylation of trk

We performed experiments to confirm that the 140-kD protein that is phosphorylated retrograde within 1 min of distal application of NGF is trk. Cultures grown under two different NGF regimes were used. In some experiments, the cultures were plated with 10 ng/ml NGF in the center compartments and 200 ng/ml NGF in the distal compartments, and after 1 wk NGF was withdrawn from the center compartments but remained at 200 ng/ml in the distal compartments. In other experiments, cultures were grown with 10 ng/ml NGF in all compartments for 2 wk. This variation in initial conditions had no effect on subsequent results.

In all experiments, cultures were given NGF-free medium for 2 h before experimental treatment. Then distal axons of control cultures were treated for 1 min with medium containing 0 ng/ml NGF, and distal axons of experimental cultures were treated for 1 min with medium containing 200 ng/ml NGF. At the end of the 1-min incubation, the cell bodies/proximal axons were immediately lysed in immunoprecipitation buffer. In each experiment lysates were harvested from equal numbers of control cultures

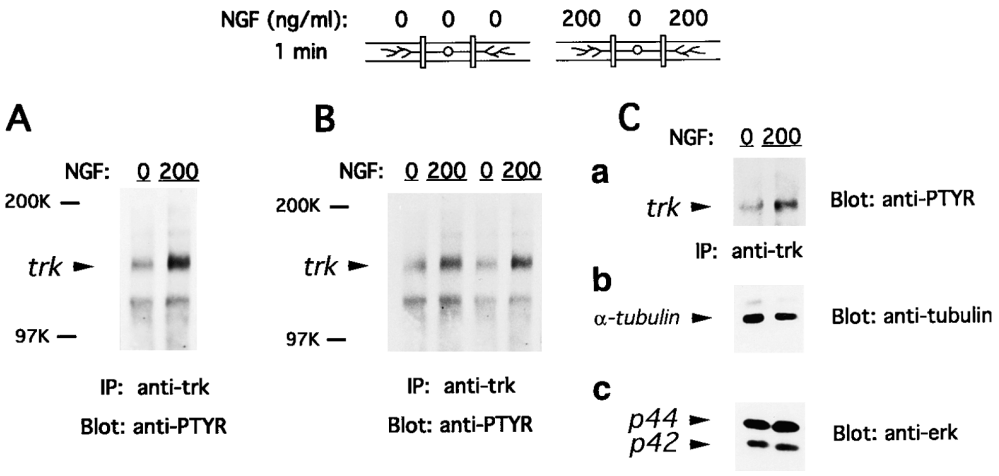

Figure 7. Retrograde phosphorylation of trk in response to distally applied NGF. (*A*) Cultures in this experiment were plated with 10 ng/ml NGF in the center compartments and 200 ng/ml NGF in the distal compartments; after 1 wk, NGF was withdrawn from the center compartments but remained at 200 ng/ml in the distal compartments. (*B* and *C*) Cultures in these experiments were supplied with 10 ng/ml NGF in all compartments for 2 wk. Cultures in all experiments were given NGF-free medium for 2 h followed by treatment for 1 min with either 0 ng/ml NGF to distal neurites (*0*), or 200 ng/ml NGF to distal neurites (*200*). In each experiment extracts of cell body/proximal axon compartments were collected from equal numbers of control and NGF-treated cultures. The number of cultures per treatment group ranged from 14–18 between experiments. In experiments in *A*, *B*, and *C a*, extracts were immunoprecipitated using anti-trk antibody and analyzed by immunoblotting with anti-phosphotyrosine antibody (*anti-PTYR*). To verify that the observed NGF-induced trk phosporylation could not have arisen from unequal loading of extracts, in experiment *C* equal aliquots of extracts from control and NGF-treatment groups removed before trk immunoprecipitation were immunoblotted with anti–α-tubulin (*C*, *b*) and anti-erk (*C*, *c*) antibodies. Neither showed an increase in the NGF treatment group. Molecular mass markers are indicated on the left of *A* and *B*.

and NGF-treated cultures. The number of cultures per treatment group ranged from 14–18 between experiments. Immunoprecipitates were prepared from the cell bodies/proximal axons' lysates and immunoblotted with anti-phosphotyrosine.

Fig. 7 shows representative results for the seven experiments that were performed. In every case, increased trk phosphorylation was observed in cell bodies/proximal axons within 1 min of application of NGF to distal axons. Fig. 7 *C* is representative of four experiments in which equal aliquots of extracts from control and NGF-treatment groups were removed before trk immunoprecipitation and immunoblotted with anti–α-tubulin (see Fig. 7 *C*, *b*) and anti-erk (see Fig. 7 *C*, *c*) antibodies. These blots confirmed that equal amounts of cellular material were harvested from control and NGF-treated groups. Tyrosine phosphorylation density scans were obtained for six of the experiments and revealed that 1-min application of NGF to distal axons resulted in a 2.4-fold increase in trk phosphorylation density in the cell bodies/proximal axons, which was highly significant ($P < 0.004$) (Fig. 6). These results clearly indicate that tyrosine-phosphorylated trk appeared in the cell bodies/proximal axons within 1 min of distal application of NGF.

Discussion

We used compartmented cultures of sympathetic neurons to investigate the appearance of retrograde tyrosine phosphorylations in cell bodies/proximal axons in response to an increase in NGF supplied to distal axons. In this way, we began to address the mechanisms of retrograde signaling by neurotrophic factors. Since the discovery that NGF is taken up by axon terminals and retrogradely transported to cell bodies (Hendry et al., 1974*a*; Stöckel et al., 1975; Claude et al., 1982; Korsching and Thoenen, 1983; Palmatier et al., 1984), it has been theorized that NGF is, itself, involved in carrying retrograde signals. The current version of the NGF transport hypothesis is that NGF binds to trk receptors on the axon terminals and is internalized by receptor-mediated endocytosis. Then, vesicles with NGF in their lumina, activating trk in their membranes, travel retrograde along the microtubule-based transport system to the cell body where activated trk initiates signaling cascades that carry the signals into the nucleus. The NGF transport hypothesis is supported by recent evidence: in PC12 cells NGF stimulates the internalization of trk into endosomes in which trk remains activated (Grimes et al., 1996), and phosphorylated trk is retrogradely transported in the sciatic nerve (Ehlers et al., 1995).

According to the above hypothesis, in the present experiments in which NGF is applied only to distal axons, the phosphorylated trk appearing in the cell bodies/proximal axons should represent the retrograde transport of activated trk bound to NGF from the distal axons. We observed that 200 ng/ml NGF applied to distal axons induced the retrograde appearance of phosphorylated trk within 1 min (Fig. 7). The retrograde phosphorylations of several other proteins were detected within 5–15 min after distal NGF administration (Fig. 4). These included several bands not observed in the distal axons (e.g., 30, 36, 38, 50, and 55 kD). Since distal compartments contain axons and the center compartments contain axons, cell bodies, and dendrites, we conclude that these bands likely represent cell body–associated proteins localized to the cell bodies and/or dendrites but absent or in low abundance in axons. This suggests that these retrograde phosphorylations reach the cell bodies. Moreover, since the proximal axons appear to be a very small fraction of material relative to the cell bodies (Fig. 1), it is unlikely that phosphorylations in the proximal axons alone would be detectable. This suggests that

all of the retrograde phosphorylations that we observed are occurring in the cell bodies.

In contrast with retrograde tyrosine phosphorylation, retrogradely transported ^{125}I-NGF was not detected for at least 30–60 min after application of 200 ng/ml ^{125}I-NGF to distal axons (Fig. 5). These data are consistent with previous evidence in compartmented cultures of sympathetic neurons indicating a 1-h lag between binding of ^{125}I-NGF to distal axons and internalization and loading of NGF onto the retrograde transport system (Ure and Campenot, 1997). These data suggest that the retrograde tyrosine phosphorylations that we observed at 1–15 min preceded the retrograde transport of the NGF that induced them.

A recent study has shown that unprimed PC12 cells treated with NGF at 4°C internalized ~37% of their surface trk receptors within 10 min and ~66% within 20 min of rewarming to 37°C (Grimes et al., 1996). This is faster internalization than that observed in distal axons of sympathetic neurons (Ure and Campenot, 1997). However, many differences in NGF responses exist between sympathetic neurons and PC12 cells. Therefore, it is quite possible that PC12 cells, especially cells that have not grown neurites or differentiated other neuronal properties, may internalize NGF into their cell bodies with different kinetics than distal sympathetic axons.

Our conclusion that NGF-induced retrograde tyrosine phosphorylations precede the arrival of retrogradely transported NGF rests upon the assumption that retrograde tyrosine phosphorylations of trk and other proteins could not have arisen by NGF diffusion across the barrier from the distal compartments and activating surface trk receptors on the cell bodies/proximal axons. Two observations rule out this possibility: analysis of the retrograde transport of ^{125}I-NGF shown in Fig. 5 indicates that, if we assume that all NGF appearing in the center compartment medium and cell extracts had resulted from diffusion, it would have produced NGF concentrations of only 2.4 pg/ml at 1 min and 28 pg/ml at 60 min. These would represent minuscule increases, especially considering that in most experiments the cell bodies and proximal axons were exposed to 10 ng/ml NGF, and these increases would amount to 0.024 and 0.28%, respectively. Also, retrograde tyrosine phosphorylations were observed in experiments in which 24 nM anti-NGF antibody was present in the center compartments to block any direct action of NGF (Fig. 4).

Fast retrograde tyrosine phosphorylations could not have arisen from contamination with distal axon lysates because several of the retrograde phosphorylations were cell body–localized proteins not observed in distal axons. When the tyrosine kinase inhibitor, K-252a, was applied to cell bodies/proximal axons, it blocked the retrograde phosphorylations without affecting tyrosine phosphorylations in the distal compartments (Fig. 3). The effectiveness of our harvesting procedures have been verified by experiments in which the neurons were completely labeled with the fluorescent dye, FM145. The axons under the barrier remained after the axons in the distal compartments, and the cell bodies and proximal axons in the center compartments had been harvested with either immunoprecipitation buffer or SDS sample buffer. Thus, the center compartment cell extracts are not contaminated with distal axon material.

The fact that the axons under the barrier remain after harvesting indicates that the barrier is sealed its entire length of ~1 mm. Our previous observations estimate the velocity of retrograde transport in compartmented cultures at 10–20 mm/h (Ure and Campenot, 1997). This is significantly higher than the 2–3 mm/h velocity of NGF retrograde transport reported for adult rat sympathetic axons in vivo (Hendry et al., 1974*a*,*b*; Johnson et al., 1978), but it is within the reasonable biological range since sensory neurons in vivo have been reported to transport NGF at 7–13 mm/h (Stöckel et al., 1975; Yip and Johnson, 1986), and sympathetic axons of the sciatic nerve have been reported to transport dopamine β-hydroxylase at 12 mm/h (Brimijoin and Helland, 1976). Using our figures, it would require 3–6 min for NGF that binds to receptors on distal axons just outside the barrier (see Fig. 1) to cross the barrier between compartments and reach the proximal axons just inside the barrier. The distal axonal material extends many millimeters from the barrier (Fig. 1); therefore, most NGF–trk receptor complexes that give rise to the trk phosphorylations in distal axons would have to travel much farther than 1 mm to reach the center compartment and be detected as retrograde phosphorylations on our Western blots. Thus, it is extremely doubtful that the tiny fraction of trk receptors on distal axons immediately adjacent to the barrier alone could produce a measurable retrograde trk phosphorylation within 1 min even if the trk receptors were internalized and transported to the center compartment within this time. These considerations rule out the possibility that a fast wave of NGF transport undetected in our ^{125}I-NGF studies could have produced the retrograde trk phosphorylation observed at 1 min.

We conclude that the appearance of tyrosine-phosphorylated trk receptors in proximal compartments within 1 min of NGF application occurred before the arrival of activated trk receptors from the distal axons. Instead we propose that this represents the phosphorylation of trk already present in the cell bodies/proximal axons at the time of distal NGF application. Since other retrogradely transported molecules are likely to travel at a similar velocity as NGF, we further believe that the extreme speed of this response precludes the mass transport of any molecular species. Rather, our results suggest that NGF binding to receptors on the surfaces of distal axons initiates a propagated signal resulting in the rapid tyrosine phosphorylation of trk proximal to the site of NGF binding to receptors on the axon surface.

Our results imply that the trk molecules phosphorylated retrograde of NGF application were not bound to NGF, but were phosphorylated by an intracellular mechanism that, in effect, bypassed the ligand binding step. Cell bodies and proximal axons have trk receptors on their surfaces that respond with tyrosine autophosphorylation when increased NGF is applied directly to them (Toma et al., 1997). Since the cell bodies/proximal axons in the present experiments were not exposed to increased extracellular NGF, most of the surface trk would not be bound to NGF. Presumably there are also intracellular organelles containing trk not bound to NGF. Any or all could be substrates for tyrosine phosphorylation by a propagated signal.

Activation of receptor tyrosine kinases without ligand binding has precedents: increased activation of trk in PC12

cells can be produced by overexpression of trk (Hempstead et al., 1992) or treatment with the ganglioside, GM1 (Ferrari et al., 1995; Mutoh et al., 1995). Rosen and Greenberg (1996) showed that Ca^{2+} influx can produce tyrosine phosphorylation of the EGF receptor in the absence of EGF. Although Ca^{2+} influx through voltage-gated Ca^{2+} channels in PC12 cells did not result in tyrosine phosphorylation of trk, the PC12 cells had no prior exposure to NGF and had not developed neuronlike properties. Therefore, when considering possible mechanisms, it would be premature to rule out Ca^{2+} as playing a role in propagating the NGF-induced retrograde phosphorylation of trk. Other speculative possibilities include phosphorylations that are self-propagated and may travel through the axon toward the cell body, or self-propagated inhibition of phosphotyrosine phosphotases.

The proteins displaying retrograde NGF-induced tyrosine phosphorylations included several low molecular weight bands not observed in distal axons, i.e., 30, 36, 38, 50, and 55 kD. Interestingly, Cabrera et al. (1996) have shown that PC12 cells given NGF and other treatments generate a 41-kD fragment of trk from which the extracellular domain has been cleaved. The truncated trk displays increased kinase activity and autophosphorylation compared with intact trk, and they suggest that generation of phosphorylated, truncated trk may be part of the NGF signal transduction mechanism in PC12 cells. Also, Zhou et al. (1995) have found a 38-kD tyrosine-phosphorylated protein in PC12 cells, which appears on antiphosphotyrosine blots in response to NGF treatment and may represent a fragment of the intracellular domain of trk.

These previous observations raise the possibility that the tyrosine-phosphorylated protein that we observed in the cell bodies in response to distal NGF may include truncated forms of trk containing the cytosolic, but not the extracellular, domain. Since we did not observe these proteins in the distal axons, it is unlikely that they are directly involved in propagating the retrograde signal. Rather, it seems that truncated forms of the cytosolic domain of NGF, if present, would be more likely part of the transduction mechanism that receives the retrograde signal after it reaches the cell body and carries it to the nucleus.

Although the appearance of the tyrosine phosphorylation of trk in Fig. 4 suggests a biphasic response of trk phosphorylation, this was not supported by densitometric analysis of all results, which indicated that retrograde trk tyrosine phosphorylation nearly doubled between 1 min and 10 min after application of NGF to distal axons (Fig. 6). However, it would be premature to rule out a biphasic response, especially since observations in PC12 cells overexpressing trk indicate that individual tyrosines are differentially phosphorylated by NGF with maximal phosphorylation of Y674 and Y675 preceding phosphorylation of the Y490 SHC binding site (Segal et al., 1996).

A question also arises as to why the propagated signal that we observed initially involved the tyrosine phosphorylation of trk at 1 min and only later involved the other proteins. This may not be the case. Since trk has five tyrosine phosphorylation sites (Kaplan and Stephens, 1994), it is possible that retrograde tyrosine phosphorylations of other proteins with fewer sites were present but below detection at 1 min after NGF administration. In fact, in a few experiments we did observe increased tyrosine phosphorylation of several other proteins at 1 min (Fig. 4 *c*).

It has been hypothesized that prolonged activation of trk and downstream second messengers by NGF may be one of the deciding factors between induction of a proliferative pathway by growth factors such as EGF and initiation of a differentiation pathway by NGF (for review see Chao, 1992). A prolonged activation of trk would also be needed to mediate the long-term promotion of neuronal survival and other trophic effects of NGF. Our mass culture experiments showed that an increase in NGF produced a rapid and prolonged increase in tyrosine phosphorylation of trk that lasted at least 24 h. In addition, we have presented data showing that trk receptors respond to increases in NGF over a broad concentration range (10–100 ng/ml; 0.4 nM–3.9 nM) (see also Belliveau et al., 1997).

These results contrast results with wild-type PC12 cells where trk tyrosine phosphorylation was maximal at 10 ng/ml NGF (Kaplan et al., 1991*b*) and returned to basal levels after only 2 h of NGF exposure (Hempstead et al., 1992; Kaplan et al., 1991*b*). However, overexpression of trk in PC12 cells produced a sustained activation of trk (Hempstead et al., 1992), similar to our observations in sympathetic neurons. Thus, the machinery for a sustained trk phosphorylation response to NGF is present in PC12 cells and likely reflects the mechanisms operative in sympathetic neurons.

The fact that sympathetic neurons respond to a broad range of NGF concentrations would enhance their ability to sense changes in the availability of NGF in the in vivo environment. While NGF has been measured in target tissues, the results have been and remain controversial (Zettler et al., 1996). Thus, the levels of NGF in the target tissues of sympathetic neurons are not established. Even if they were established, it is likely that extracellular NGF is not uniformly distributed in the target cell environment; e.g., it is possible that NGF release sites could be localized near axon terminals and expose them to a much higher NGF concentration than the target tissue average. In this regard, it is relevant that glutamate released by hippocampal neurons reaches concentrations as high as 1.1 mM in the synaptic cleft (Clements et al., 1992). Thus, although we used a high NGF concentration of 200 ng/ml NGF in our experiments to saturate the NGF receptors on the distal axons, it cannot be concluded a priori that this concentration is beyond the biological range. In fact, previous experiments investigating the induction of mRNAs for Tα1 α-tubulin, tyrosine hydroxylase, and p75 neurotrophin receptor in sympathetic neurons in mass culture show that gene expression increases over the range of 10–200 ng/ml NGF (Ma et al., 1992). Moreover, experiments also showed that the retrograde induction of gene expression when 200 ng/ml NGF was only applied locally to distal axons was not a maximal response since substantial additional increases were observed when 200 ng/ml NGF was also applied to the cell bodies and proximal axons (Toma et al., 1997). Thus, the concentrations used in the present experiments were in the biologically effective range, and application of 200 ng/ml NGF to distal axons did not saturate the ability of the neurons to respond to NGF.

While our results indicate that retrograde signaling by NGF must include other mechanisms besides the retro-

grade transport of NGF-containing vesicles, they by no means rule out that signals are also carried by NGF-containing vesicles. In fact, previous results indicate that NGF is not degraded during retrograde transport and accumulates in the neuronal cell bodies where it resides with a $t_{1/2}$ of ~3 h, which is consistent with a retrograde signaling role (Ure and Campenot, 1997). On the other hand, under steady state transport conditions, only a small fraction of the axon-bound NGF was delivered to the cell bodies each hour, and a far greater fraction of the neuron-associated NGF was bound to distal axons than was present in the cell bodies (Ure and Campenot, 1997). This is consistent with recent in vivo observations suggesting that a large fraction of NGF in target tissues may be associated with sympathetic axons (Zettler et al., 1996). NGF bound to axonal receptors undoubtedly has other functions besides retrograde signaling, e.g., the activation of local signaling pathways that regulate neurite growth (for review see Campenot, 1994) and presumably regulate other local functions of the axon. However, the present results raise the possibility that a major function of NGF bound to axonal trk receptors is to give rise to intracellular signals that reach the cell body by mechanisms not involving NGF transport.

In conclusion, we have presented evidence that application of NGF to distal axons of rat sympathetic neurons in compartmented cultures results in the appearance of tyrosine-phosphorylated trk and other proteins in the cell bodies before the arrival of the NGF that induced them. These data do not support the concept of retrograde transport of NGF and associated signaling molecules as the only mechanism of retrograde signaling along axons. Our data suggest rather that binding of NGF to trk receptors on axon terminals generates intracellular tyrosine phosphorylations of trk and other proteins by a rapid propagation mechanism. Our results have broad implications for the mechanisms of retrograde signaling by all neurotrophic factors, raising the possibility that many kinds of retrograde signals may reach the neuronal cell bodies without the retrograde transport of signaling molecules.

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