Nerve Growth Factor (NGF)-induced Calcium Influx and Intracellular Calcium Mobilization in 3T3 Cells Expressing NGF Receptors*

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NGF, the first discovered and best characterized member of this family, is required for the survival and development of sympathetic and sensory neurons in the peripheral nervous system (1, 2). It also acts on specific populations of neurons in the central nervous system (3–5) and on cells of the adrenal medulla (6, 7).

Two classes of neurotrophin receptors have been identified, usually designated high affinity receptors and low affinity receptors. Many of the biological activities of the neurotrophins are mediated by their binding to and activation of high affinity receptor tyrosine kinases (8). These receptors are encoded by the trk gene family. NGF preferentially binds to p140

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† Shortly after revision of this manuscript, Dr. Gordon Guroff, Chief of Section on Growth Factors, NICHHD, and §Pathology Section, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

The neurotrophins have been implicated in the acute regulation of synaptic plasticity. Neurotrophin-stimulated presynaptic calcium uptake appears to play a key role in this process. To understand the mechanism of neurotrophin-stimulated calcium uptake, the regulation of calcium uptake and intracellular mobilization by nerve growth factor (NGF) was investigated using NIH 3T3 cells stably transfected with either the high affinity NGF receptor p140

From the ‡Section on Growth Factors, NICHD, and §Pathology Section, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

The neurotrophins are required for the survival and differentiation of many types of neurons during development. The neurotrophin family consists of five structurally and functionally related polypeptides and includes nerve growth factor (NGF),1 brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), neurotrophin 4/5 (NT-4/5), and neurotrophin 6 (NT-6).

The Abbreviations used are: NGF, nerve growth factor; p140

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Brain to store information. And these neurotrophin-dependent changes in neurotransmitter release are dependent, in turn, on increases in presynaptic calcium uptake (27). Thus, it seems likely that the uptake of calcium is a crucial element in both the long and the short term actions of the neurotrophins.

A possible model for these neurotrophin-dependent increases in calcium uptake may be the PC12 cell (28, 29). Previous studies have shown that NGF causes increases in calcium uptake into PC12 cells (30) and that this increased uptake seemed to be mediated by unique calcium channels (31). It also appeared that an NGF-induced phosphorylation, perhaps of the calcium channel itself, is required for this increased uptake (31). The strength of the NGF-induced increase in calcium uptake is dependent on the intracellular calcium concentration (32); calcium uptake is greater in cells depleted of calcium and weaker in cells in which calcium levels are raised. Protein kinase C appears to participate in the process of NGF-induced calcium uptake (33). K-252a, a kinase inhibitor that blocks the actions of NGF on PC12 cells (34), mimics the action of NGF on calcium uptake (30, 35) and may employ the same signaling elements as does NGF, including protein kinase C and, perhaps, the high affinity NGF receptor itself.

In a previous study it was shown that NGF induced an increased uptake of calcium into 3T3 cells stably transfected with either p140\textsuperscript{trk} or p75\textsuperscript{NGFR} (36). In the present study we used confocal microscopy to investigate further the alterations in calcium levels produced by NGF in these two separate cell populations.

**EXPERIMENTAL PROCEDURES**

**Materials**—NGF and rat collagen type II were purchased from Collaborative Biomedical Products (Bedford, MA). BDNF was obtained from Gennzyme (Cambridge, MA). K-252a was kindly provided by Dr. Y. Matsuda (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan). The monoclonal anti-phosphotyrosine antibody 4G10 was a product of Upstate Biotechnology, Inc. (Lake Placid, NY). The polyclonal anti-TrkA (C14) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and polyclonal anti-p75 antibody was obtained from Promega (Madison, WI).

**Cell Culture and Transfection—**3T3 transfectants were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and 100 µg of streptomycin and 100 units of penicillin (Life Technologies, Inc.) per ml. The pCMV-trkA expression plasmid (36) and the pCMV5-p75 expression plasmid (kindly provided by Dr. Moses Chao) were transfected separately into NIH 3T3 cells by the calcium phosphate method (36). The stable clones WT11, L2, and L9 were selected in 0.5 mg/ml G418 and were used in the present studies. For infection, 3T3 transfectants were grown in calcium-free media (36). The stable clones KD215 and KD217, a mutation of Lys-538 to Asn (K538N) was introduced into pLXSN-trkA with the Quickchange site-directed mutagenesis kit (Stratagene).

**Immunoblotting and Immunoprecipitation—**Cells were treated with a lysis buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% deoxycholate, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM sodium vanadate, and 50 µM sodium fluoride at 4 °C for 30 min. Insoluble material was removed by centrifugation, and equal amounts of lysates were used for either immunoblotting or immunoprecipitation according to the methods previously described (36). For kinase-deficient trkA clones, KD215 and KD217, a mutation of Lys-538 to Asn (K538N) was introduced into pLXSN-trkA with the Quickchange site-directed mutagenesis kit (Stratagene).

**Measurement of Intracellular Calcium—**3T3 transfectant cells were plated into collagen- and polylysine-coated two-well chambers (Nalge) 1 day before each experiment. The cells were loaded with 4 µM Fluo-3-AM (Molecular Probes, Eugene, OR) for 1 h at 37 °C with slight modifications of the methods previously described (32). The cells were then washed twice with 1-ml portions of fresh medium and immediately used for experiments. The fluorescence of intracellular Fluo-3 was quantified by confocal laser scanning fluorescence microscopy (Leica TCS4D, Leica Lasertechnik Heidelberg, Germany) using excitation and emission wavelengths of 485 and 525, respectively. Gray scale images were collected at different time points before and up to 10 min after the addition of NGF by using a 512 × 512 pixel format and archived as tiff image files for later analysis. The intensity of the fluorescence in individual cells was measured using Leica quantification software. For each treatment, the relative intensity of three or more typical cells was measured, and the mean value of the fluorescence per unit area of the cell was calculated.

**RT-PCR Analysis of Calcium Channel Subunit Transcripts—**Total RNA was isolated from NIH 3T3 cells and PC12 cells by RNA-STAT-60 (Tel Test, Inc., Friendswood, TX). RT-PCR reactions were carried out by Superscript One-step RT-PCR system (Life Technologies, Inc.) for 35...
cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 30 s. The primers used for specific isoforms of α₁ subunits of calcium channels, as described previously (40), were as follows: rat α₁A-forward 5′-CCAGTCTGTGGAGATGAGAGAAATGGG-3′ (residues 6042–6068, GenBank™ accession number M64373); α₁A-reverse 5′-TTTGGAGGGCAGGTCACCCGATTG-3′ (residues 6412–6435); rat α₁B-forward 5′-GCCGTCTCAGCCGCGGCCTTTCT-3′ (residues 6668–6690, GenBank™ accession number M92905); α₁B-reverse 5′-CAAAAGGTGAGTGTATCCTCAGGC-3′ (residues 6810–6832); rat α₁C-forward 5′-AGAAGAGAAGAGACTGGC-3′ (residues 3064–3090 in rat α₁C rbc-I, GenBank™ accession number M67516); α₁C-reverse 5′-CGGGGGCGTGGGCCCACAGGCATCTCG-3′ (residues 3307–3333). These primers have 100% homology with the corresponding sequences of mouse origin. The RT-PCR products were resolved on 4% agarose gels, and individual PCR fragments were isolated, cloned in pCRII vector (Invitrogen), and sequenced.

RESULTS

The expression of human p140<sup>trk</sup> and of p75<sup>NGFR</sup> in 3T3-Trk and 3T3-p75 transfectants, respectively, was analyzed by immunoblotting and immunoprecipitation. Both p140<sup>trk</sup> and p75<sup>NGFR</sup> were highly expressed in the appropriately transfected 3T3 cells but not in the parent cells (Fig. 1A). Indeed, p140<sup>trk</sup> is seen as a doublet, as it is in other p140<sup>trk</sup>-containing cell lines, the lower band representing an underglycosylated precursor (41–43). As noted previously (36), the WT 11 and WT 108 clones of 3T3-Trk cells have 3–4-fold more p140<sup>trk</sup> receptors than do PC12 cells; the 3T3-p75 clones have about 50% as many p75<sup>NGFR</sup> receptors as do PC12.

To examine NGF-induced p140<sup>trk</sup> phosphorylation and downstream signaling pathways, WT11 and WT108 cells were treated with 100 ng/ml NGF for 5 min. NGF induced a strong phosphorylation of p140<sup>trk</sup> (Fig. 1B) and an activation of mitogen-activated protein kinase (data not shown) in both clones. NGF failed, however, to induce either p140<sup>trk</sup> phosphorylation (Fig. 1B) or mitogen-activated protein kinase activation (data not shown) in either KD215 or KD217, clones transfected with a kinase-deficient mutant of p140<sup>trk</sup>. K-252a, a tyrosine kinase inhibitor that acts on the Trk family of neurotrophin receptors in PC12 cells (34), blocked NGF-induced p140<sup>trk</sup> phosphorylation in both WT11 and WT108 cells (Fig. 1C). These results suggest that transfection of human p140<sup>trk</sup> into NIH 3T3 cells results in activation of functional NGF receptors coupled to appropriate downstream signaling pathways.

Previous studies have shown that NGF induced <sup>45</sup>Ca<sup>2+</sup> influx into PC12 cells (30) and into both 3T3-Trk and 3T3-p75 cells as well (36). To explore this observation further and to
determine the subcellular distribution of the increased intracellular calcium, the
increase in intracellular calcium in both WT11 (Fig. 2A) and
WT108 cells (data not shown). The time of peak increase is
about 4 min after NGF treatment (Fig. 2C). The intracellular
calcium level began to decrease after about 6 min but remained
above basal for at least 10 min after NGF treatment. Nuclear
calcium levels were somewhat higher than those in the cytosol.

Recently it has been shown that NGF induces intracellular
calcium mobilization in C6 glioma cells transfected with
p140<sup>Trk</sup> receptors (45). In order to examine the effects of NGF
on both calcium influx and calcium mobilization, regular calci-
um-containing medium was replaced with either calcium-free
medium or regular medium containing 2 mM EGTA. In WT11
cells in calcium-free medium, NGF also induced an initial in-
crease in intracellular calcium levels (Fig. 2B). The peak was
reached in about 3–4 min, and the levels returned to basal in 8
min (Fig. 2D). Ligand-induced increases in intracellular cal-
cium levels in calcium-free medium have been attributed to
mobilization of intracellular stores. The persistent increased
levels above basal seen in calcium-containing, but not in calci-
um-free, medium are thought to be due to uptake from extra-
cellular sources. This observation suggests that activation of
p140<sup>Trk</sup> by NGF can induce both calcium influx and release of
calcium from internal stores.

In order to define the role of the p140<sup>Trk</sup> receptor in this
process, two kinase-deficient clones, KD215 and KD217, were
used. NGF did not induce any increase in intracellular calcium
concentrations in KD217 cells (Fig. 3A). Preincubation with
500 nM K-252a for 30 min before the ad-
dition of NGF.

In the 3T3-p75 clone L9 (Fig. 4A), NGF also induced a sig-
nificant increase in intracellular calcium levels. The peak of the
increase was at about 6 min after NGF treatment (Fig. 4A).
There appeared to be an increase in nuclear calcium levels in
these cells as well, but the increase was no greater than that
seen in the cytoplasm.

In 3T3-p75 cells, NGF induced an increase in intracellular
calcium in the presence of extracellular calcium but had no
effect in the absence of extracellular calcium (Fig. 4B and D).
This suggests that activation of p75<sup>NGFR</sup> supports only calcium
uptake but not calcium mobilization from intracellular stores.
Preincubation with K-252a did not block the NGF-induced
increase in intracellular calcium in 3T3-p75 cells (data not
shown).

Signaling by the p75<sup>NGFR</sup> appears to involve the sphingomy-
elin cycle and the second messenger ceramide (18). The addi-
tion of C<sub>2</sub>-ceramide to 3T3 cells resulted in an increase in
intracellular calcium (Fig. 5A). Consistent with this, ceramide
treatment produced no increase in intracellular calcium in
calcium-free medium (Fig. 5B). So the addition of ceramide
appears to mimic the action of NGF on p75NGFR; it produces an increase in calcium uptake but no increase in calcium mobilization.

Previous experiments (30, 35) have shown that K-252a alone mimics the actions of NGF on $^{45}$Ca$^{2+}$ uptake, as it does on neurotransmitter release (46), in PC12 cells, and that uptake appears to be mediated by the p140$^{trk}$ receptor (35). Treatment of 3T3-Trk cells with K-252a produced a marked increase in calcium levels in the cells (Fig. 6A) and that increase appeared, as does the increase produced by NGF in these cells, to be comprised of an increase in calcium uptake and an increase in calcium mobilization as well, since the increase in calcium-free medium was less than that in complete medium and returned to base line readily (Fig. 6B). Treatment of 3T3-p75 cells with K-252a in calcium-containing medium produced no increase in calcium levels (Fig. 6C). Thus, the actions of K-252a on these cells appears to be mediated by the p140$^{trk}$ receptor, as it does in PC12 cells (35).

Since recent data indicate that NGF stimulates calcium uptake through l-type calcium channels in PC12 cells,2 l-type calcium channels were sought in these 3T3 transfectants. PCR analysis showed the presence of transcripts for calcium channel subunits $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1C}$ in both 3T3 cells and PC12 (Fig. 7). This last, $\alpha_{1C}$, is the $\alpha$ subunit found in l-type calcium channels. As further proof, it was shown that BayK 8644, an l-type calcium channel agonist, stimulates calcium uptake into these cells and that this uptake is inhibited by nifedipine, an l-type channel antagonist (Fig. 8, A and B). When nifedipine was added to cells stimulated with NGF, inhibition was seen with both 3T3-Trk (Fig. 9, A and B) and 3T3-p75 cells (Fig. 10, A and B), l-type indicating that binding to either p140$^{trk}$ receptors or p75NGFR receptors will activate nifedipine-sensitive l-type calcium channels.

**DISCUSSION**

The effect of NGF on calcium uptake, especially in PC12 cells, has been a contentious subject for some 2 decades. It was first reported in 1978 (47) that NGF treatment produces a small increase of calcium release from PC12 cells. However, this observation was not supported by subsequent studies (48). More recently, the direct effects of NGF on the intracellular calcium levels of NGF-responsive cells have been reinvestigated and have led to the finding that NGF produces a small increase of calcium release from PC12 cells.
and rapid increase in intracellular calcium concentrations in PC12 cells (49, 50) and that certain of the effects of NGF depend upon the presence of extracellular calcium (51, 52).

One of the effects of NGF on PC12 cells is to induce catecholamine release, and this release is dependent upon the presence of extracellular calcium (46). By using an assay that measures the uptake of radioactive calcium into PC12 cells, it was further shown that NGF induced a small but rapid increase in the uptake of calcium (30). The increase of calcium uptake is maximal after 5 min of NGF treatment and gradually disappears after 15 min. This observation was further supported by studies using the fluorescent dye, Fluo-3 (32). Those experiments showed that NGF treatment of PC12 cells produces a rapid and transient uptake of divalent cations and an increase in their intracellular level as well. Most significant, this increase was stronger when the calcium levels of the cells were low and weaker when it was high. This suggests that the effects of NGF are to modulate calcium levels in either direction, depending on the needs of the cells at the time.

In order to identify potential calcium channels involved in NGF-induced calcium uptake into PC12 cells, a number of calcium channel blockers were used (31). Nifedipine, a blocker of L-type calcium channels, partially inhibited NGF-induced calcium uptake, suggesting that L-type calcium channels may be at least partly responsible for the NGF-induced calcium uptake. This suggestion has been confirmed through whole cell patch clamping studies.2

Both p140<sup>trk</sup> and p75<sup>NGFR</sup> are present on PC12 cells. Experiments with <sup>45</sup>Ca<sup>2+</sup> using 3T3-Trk and 3T3-p75 cells have shown that both of these receptors will support increased calcium uptake (36). These experiments highlight an additional Trk-independent role for the low affinity NGF receptor, p75<sup>NGFR</sup>, and may be related to some recent observations on NGF-induced neurotransmitter release; the NGF-induced re-
lease of dopamine from striatal neurons is mediated by p75NGFR (53), and NGF enhances the depolarization-evoked release of glutamate and acetylcholine from visual cortex synaptosomes through p75NGFR as well as through p140^src^ (54).

The present studies also show that both receptors will mediate increases in intracellular calcium levels. NGF induces both increased calcium uptake and increased calcium mobilization through p140^src^ but only increased calcium uptake through p75NGFR. In this regard it should be noted that, although neurotrophin-mediated neurotrophin release through p140^src^ is supported by the mobilization of intracellular calcium stores (55), more recent data show that p75NGFR also can promote neurotrophin-mediated neurotrophin release (56). Since the present data show that p75NGFR does not support the mobilization of intracellular calcium stores, it is reasonable to conclude that NGF-induced calcium uptake from the extracellular compartment can also support neurotrophin-induced neurotrophin release.

The response of p140^src^ requires intracellular signaling, since kinase-deficient mutants do not support either function. The response of p75NGFR also appears to require intracellular signaling, since C2-ceramide produces an increase in calcium uptake, and it is known that the p75NGFR can signal through the ceramide pathway (18). The ability of p140^src^ to promote the mobilization of intracellular calcium is consistent with what is known about its signaling, i.e. its ability to activate phospholipase C^y^ (12), which produces inositol 1,4,5-trisphosphate, which, in turn, activates one of the receptors controlling intracellular calcium release. No such mobilizing function has been ascribed to p75NGFR.

The data suggest that both p140^src^ and p75NGFR support increases in nuclear calcium as well as in cytoplasmic calcium. This is significant because it has recently been shown that increases in nuclear calcium levels have different biological consequences than do increases in cytoplasmic calcium (57).

It is important to know what mechanism is activated by NGF to bring calcium into the cell, because it is clear that the mechanism by which calcium enters determines its actions within the cell (58). In the case of the receptors studied here both appear to be linked to L-type calcium channels. L-type channels are clearly present on the cells, and an antagonist of L-type channels inhibits the actions of NGF through either of its receptors. Although this may likely be true in this cell type, it is important to note that nifedipine also inhibits, at least partially, the uptake of 45Ca^2^ into PC12 cells (31), and the application of NGF or of BDNF to PC12 cells produces increases in voltage-activated calcium currents through L-type channels (59).

In summary, neurotrophins are critical for neuronal survival, neuronal differentiation, neuronal protection, and synaptic remodeling. For many of these long and short term functions of the neurotrophins, the control of calcium levels is crucial. In particular, the neurotrophin-mediated presynaptic uptake of calcium, which leads to the neurotrophin-stimulated presynaptic release of neurotransmitter, appears to be a key element in synaptic plasticity and long term potentiation. The detailed mechanism(s) by which the neurotrophins stimulate calcium uptake and modulate intracellular calcium levels provides an intriguing avenue for study.

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