Both p140<sup>trk</sup> and p75<sup>NGFR</sup> Nerve Growth Factor Receptors Mediate Nerve Growth Factor-stimulated Calcium Uptake* (Received for publication, November 4, 1996, and in revised form, January 9, 1997) Hao Jiang, Danielle St. Ulme, Geneva Dickens, Alia Chabuk, Miriam Lavarrada, Philip Lazarovici‡, and Gordon Guroff§ From the Section on Growth Factors, NICHD, National Institutes of Health, Bethesda, Maryland 20892

Human p140<sup>trk</sup> and p75<sup>NGFR</sup> were transfected separately into 3T3 cells. Nerve growth factor stimulates calcium uptake into both transfectants but not into untransfected 3T3 cells. p140<sup>trk</sup> cells were stimulated maximally by 25 ng/ml; 100 ng/ml was submaximal for p75<sup>NGFR</sup> cells. K-252a inhibits the effect of NGF on p140<sup>trk</sup> cells but not on p75<sup>NGFR</sup> cells; brain-derived neurotrophic factor stimulates calcium uptake in p75<sup>NGFR</sup> cells but not in p140<sup>trk</sup> cells. The data suggest that both nerve growth factor receptors could be involved in the nerve growth factor-mediated actions of calcium on its target cells: neuronal survival, neuronal protection, and synaptic plasticity.

Nerve growth factor (NGF),<sup>1</sup> a member of the neurotrophin family, is required for the survival and development of sympathetic and sensory neurons in the peripheral nervous system (1, 2). It also acts on cholinergic neurons in the central nervous system (3–5), on chromaffin cells of the adrenal medulla (6, 7), and on a number of tumor cell lines as well (8, 9). The most dramatic and informative in vitro responses to NGF are observed in the pheochromocytoma cell line PC12, which has been used extensively as a model for investigations of NGF-induced neuronal differentiation (10, 11). Although there is substantial information about the mechanism by which NGF causes profound and global changes in cell phenotypes, the identification of specific second messengers responsible for specific biological actions of NGF has proven difficult. NGF-induced changes in a number of these second messengers, such as cAMP (12), GMP (13), calcium (14), phosphoinositides (15), arachidonic acid (16, 17), and glycosphosphatidylinositol metabolites (18), have been reported in PC12 cells and other NGF-responsive cells.

Perhaps the multiple roles of calcium in neurotrophin action are as well understood as any. Neuronal survival appears to depend upon the maintenance of sufficiently high levels of intracellular calcium (19), and when such levels are sufficient, neurons normally dependent on NGF for survival become NGF-independent (20), suggesting a role for NGF in keeping calcium levels from falling too low. On the other hand, when neurons sustain oxygen deprivation or hypoglycemia, the damage that ensues is due at least in part to extremely elevated intracellular calcium levels. Under such conditions, neurons can be protected by neurotrophins, which also keep calcium levels low (21), suggesting a role for neurotrophins in keeping calcium levels from rising too high. More recently, it has been shown that neurotrophins can mediate synaptic plasticity (22), the more or less permanent change in synaptic efficiency that appears to underlie long term potentiation, thought by some to be the biochemical cognate of learning and memory. Synaptic plasticity appears to depend on neurotransmitter release, dependent, in turn, upon neurotrophin-induced calcium influx (23). So both long and short term actions of the neurotrophins appear to involve regulation of intracellular calcium levels, at least in part through regulating calcium uptake.

Indeed, it has been shown directly that NGF can stimulate a small, rapid increase in intracellular calcium concentration in PC12 cells (24–26) and that this increase is accompanied by an NGF-induced increase in calcium uptake (27). Increased calcium uptake appears to be required for NGF-induced increases in neurotransmitter release (28). The molecular mechanism of NGF-induced calcium uptake is not well understood. The pharmacological profile of NGF-induced calcium uptake in PC12 cells suggests that a unique calcium channel may be involved (29) in this process and that phosphorylation of this channel may be required because introduction of phosphatase prevents increased calcium uptake (29). Beyond that, little information is available.

NGF binds to two separate receptors on its target cells, a site with tyrosine kinase activity, p140<sup>trk</sup>, termed by some the high affinity receptor, and p75<sup>NGFR</sup>, which appears to signal through the ceramide pathway, thought by some to have lower affinity for NGF. Most of the physiological actions of NGF are mediated by p140<sup>trk</sup> (30); p75<sup>NGFR</sup> has recently been associated with regulated cell death (31). To determine which receptor is responsible for NGF-induced calcium uptake, we stably transfected the human p140<sup>trk</sup> and p75<sup>NGFR</sup> separately into NIH3T3 fibroblast cells, which do not have endogenous NGF receptors, and examined the effects of NGF on the uptake of radioactive calcium into these transfectants.

EXPERIMENTAL PROCEDURES

Plasmids and Transfection—Full-length human p140<sup>trk</sup> cDNA sequence was kindly provided by Dr. Andrea Levi and inserted into the pRCMV vector (Invitrogen). pCMV5-p75 plasmid containing human p75<sup>NGFR</sup> was kindly proved by Dr. M. V. Chao. The expression vectors containing either human p140<sup>trk</sup> or human p75<sup>NGFR</sup> were transfected into 3T3 fibroblasts using calcium phosphate, and stable clones were selected. Stable clones expressing p140<sup>trk</sup> or p75<sup>NGFR</sup> were screened by Western blot analysis using either anti-trk203 antibody, a gift from Dr. David Kaplan, or anti-p75<sup>NGFR</sup> antibody (Promega) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 μg/ml of streptomycin, 100 units/ml of penicillin, and 200 μg/ml of G418 (Life Technologies, Inc.). The presence of RNA transcripts was further confirmed by reverse transcription-polymerase chain reaction using specific human p140<sup>trk</sup> and p75<sup>NGFR</sup> primers.

Western Blot Analysis—The cells were harvested by scraping on ice and were washed twice with ice-cold phosphate-buffered saline. The cell
pellet was collected by centrifugation and lysed for 30 min on ice in 0.5–1 ml of lysis buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% deoxycholate, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 1 μM aprotinin). The total cell lysates were collected by centrifugation at 10,000 rpm for 10 min, and the protein concentration of each sample was determined by the Bio-Rad protein assay. Equal amounts of cell lysate (50 μg) were applied to 8% SDS-polyacrylamide gels. After gel electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Millipore), and the membranes were incubated with blocking buffer for 1 h and then transferred to the appropriate antibody overnight at 4°C. The Western blots were analyzed using an enhanced chemiluminescence protein detection kit (Tropix) according to the manufacturer’s instructions.

**RESULTS AND DISCUSSION**

In the course of experiments with PC12 cells (27, 29, 38), it became clear that the interaction of NGF with p140<sup>trkA</sup> was not sufficient to explain all the observations. Small but persistent increases in calcium uptake were seen when PC12mn5 cells, a PC12 variant with little or no p140<sup>trk</sup> receptors, were treated with NGF. In addition, tyrosine kinase inhibitors were not able to inhibit the NGF effect on calcium uptake completely. The significance of the p75<sup>NGFR</sup> was predicted by experiments with PC12 cells and BDNF, which interacts with p75<sup>NGFR</sup> but not with p140<sup>trk</sup>. A combined average of five such experiments, each done in triplicate, showed that BDNF produced an increase of 19 ± 6% in calcium uptake with a p value of <0.001; in another series of four experiments, each also done in triplicate, BDNF produced an increase of 19 ± 15% with a p value of <0.05, whereas NGF gave an increase of 27 ± 8.4% with a p value of <0.001.

To provide definitive information on which NGF receptor was involved in calcium uptake, the two NGF receptors were transfected separately into 3T3 cells, which have no endogenous NGF receptors. Two stable clones were used for the present studies. One of these clones, 3T3-trkA-WT.11, has 3–4-fold more p140<sup>trk</sup> receptors than do PC12 cells. NGF can stimulate

**FIG. 1.** Western blot analysis of 3T3-trk and 3T3-p75 cells. Equal amounts of cell protein (50 μg) were used for Western blotting analysis as described under “Experimental Procedures.” p140<sup>trk</sup> and p75<sup>NGFR</sup> protein were detected using anti-trk 203 and anti-p75 antibodies, respectively.

**FIG. 2.** NGF-induced calcium influx in 3T3-trkA and 3T3-p75 cells. The transfectant cells were grown and calcium uptake experiments were performed as described under “Experimental Procedures.” After incubation in calcium-free, serum-free, antibiotic-free medium for 30 min, different concentrations of NGF were added, and the cells further were incubated for 4 min. The numbers in parentheses indicate the number of individual experiments, each done in triplicate; asterisks indicate p < 0.05 compared with control.

**FIG. 3.** The effect of K-252a on NGF-stimulated calcium influx in 3T3-trkA and 3T3-p75 cells. The transfectant cells were grown and calcium uptake experiments were performed as described under “Experimental Procedures.” The cells were incubated for 30 min in calcium-free medium, pretreated with 200 nM K-252a for 30 min at 37 °C, and stimulated with NGF (100 ng/ml) for 4 min at 37 °C. The numbers in parentheses indicate the numbers of individual experiments, each done in triplicate; single asterisks indicate p < 0.05 compared with control (CON), and the double asterisk indicates p < 0.05 compared with control.

**FIG. 4.** The effect of K-252a on calcium influx in 3T3-trkA and 3T3-p75 cells. The transfectant cells were grown and calcium uptake experiments were performed as described under “Experimental Procedures.” The cells were incubated for 30 min in calcium-free, serum-free, antibiotic-free medium and stimulated with K-252a (200 nM) or NGF (100 ng/ml) for 4 min at 37 °C. The numbers in parentheses indicate the numbers of individual experiments, each done in triplicate; asterisks indicate p < 0.05 compared with control (CON).
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both p140NFκB autophosphorylation and Erk phosphorylation in 3T3-trkA cells (data not shown). The presence of p140NFκB receptors on the cell surface was also confirmed by 125I-NGF binding and immunoblotting using the membrane fraction from 3T3-trkA cells. The other clone, 3T3-p75-L2, has about 50% less p75NFGR receptors than do PC12 cells but many more than in PC12nrr5 cells (Fig. 1).

Using the conditions under which NGF stimulates calcium uptake in PC12 cells, we estimated dose-dependent NGF-induced calcium uptake in both 3T3-trkA and 3T3-p75 cells. In both transfectants, but not in untransfected 3T3 cells, NGF can induce increased calcium uptake (Fig. 2) to a level similar to that observed in PC12 cells (27), although the stimulation in replicative cultures was somewhat variable, usually between 25 and 50% but sometimes as high as 100%. At low concentrations, NGF seems to induce calcium uptake more effectively in 3T3-trk cells than in 3T3-p75 cells, perhaps reflecting the different binding affinities of NGF for the p140NFκB and the p75NFGR receptors.

K-252a, an alkaloid-like kinase inhibitor isolated from the culture broth of Nocardiosis (32–34), has been shown to specifically inhibit many of the actions of NGF in PC12 cells, including NGF-induced neurite outgrowth (35, 36) and the tyrosine kinase activity of p140NFκB (37). Pretreatment of 3T3-trkA cells with 200 nM K-252a for 30 min can specifically block NGF-induced calcium uptake (Fig. 3). However, a similar pretreatment of 3T3-p75 cells has no effect on NGF-induced calcium uptake.

In PC12 cells, K-252a treatment produces a small increase in the uptake of radioactive calcium within 3–4 min (27), and this increased uptake appears to involve p140NFκB (38). Consistent with these previous data, K-252a treatment of 3T3-trkA cells stimulated a small increase in the uptake of radioactive calcium but had no effect in 3T3-p75 cells (Fig. 4). K-252a-induced calcium uptake is smaller than NGF-induced calcium uptake in 3T3-trk cells as it is in PC12 cells (27).

BDNF is another member of the neurotrophin family, which, as stated above, can bind to p75NFGR but not to p140NFκB. Because NGF can induce calcium influx in 3T3-p75 cells, we examined the effect of BDNF on calcium influx in both 3T3-trk and 3T3-p75 cells. BDNF can increase calcium influx into 3T3-p75 cells, as does NGF, but has no effect on calcium influx into 3T3-trk cells (Fig. 5).

The data presented here clearly show that NGF can increase calcium uptake through both NGF receptors. These data are consistent with those in a recent report suggesting that NGF can increase neurotransmitter release from striatal neurons through the p75NFGR receptor (39). Clearly, the NGF-induced increases in calcium uptake in 3T3 cells have no biological relevance, the NGF receptors being introduced artificially. But increases of comparable magnitude in PC12 cells are absolutely required for the NGF-induced release of catecholamine neurotransmitters (28). Also there is a growing awareness that capacitative calcium entry plays a central role in many aspects of cell signaling (40), and the amounts of calcium entering the cells by this route appear to be very small indeed.

It is important to determine the mechanism(s) by which these receptors act on calcium uptake, because there is evidence that the mechanisms by which calcium enters the cell determines its actions in those cells (41). In any case, the present data suggest that both high and low affinity NGF receptors can participate in the calcium-mediated actions of the neurotrophins.

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