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# Nerve Growth Factor Enhances Neurotransmitter Release from PC12 Cells by Increasing $Ca^{2+}$-Responsible Secretory Vesicles through the Activation of Mitogen-Activated Protein Kinase and Phosphatidylinositol 3-Kinase[1]

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**Neurotrophins play important roles in the differentiation and survival of neurons during development, and in the regulation of synaptic transmission in adult brain. Brief treatment with nerve growth factor (NGF) enhances depolarization and ionomycin-induced dopamine and acetylcholine release from PC12 cells. The enhancing effect appears very quickly and reaches a plateau 10–15 min after application. NGF also enhances hypertonic solution–induced dopamine release, and increases the amount of dopamine released from membrane-permeabilized PC12 cells in the absence of MgATP, suggesting that NGF enhances neurotransmitter release by increasing the number of $Ca^{2+}$-responsive secretory vesicles. The activation of Trk receptors is essential for NGF action, since K252a abolishes the NGF-induced potentiation of dopamine release and brain-derived neurotrophic factor enhanced ionomycin-induced release only in TrkB-expressing cells. NGF-mediated potentiation of dopamine release is completely abolished by wortmannin, a PI 3-kinase inhibitor, and by U0126 and PD98059, MAP kinase kinase inhibitors, indicating that the activation of PI 3-kinase and MAP kinase pathways is essential for NGF action. These findings suggest that NGF regulates neurotransmitter release through the activation of TrkA receptors, possibly by increasing the number of secretory vesicles in a readily releasable pool.**

**Key words: NGF, neurotransmitter release, MAP kinase, PI 3-kinase, TrkA.**

Activity-dependent modification of synaptic transmission, or synaptic plasticity, is regarded as the cellular basis of learning and memory (*1*). Various endogenous factors are believed to be involved in synaptic plasticity, however, the precise mechanisms are still unclear. Neurotrophins, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5, are members of a family of proteins that plays important roles in the survival and differentiation of many types of neurons during development (*2–4*). Recently, these neurotrophins and their receptors were found to be expressed in adult brain, and to participate in activity-dependent synaptic plasticity and memory formation (*4–7*). Among the neurotrophins, BDNF has been most extensively studied for its role in synaptic plasticity. BDNF induces long lasting potentiation of synaptic transmission by various mechanisms, including increasing either the expression levels of synaptic proteins or the number of neurotransmitter releasing sites (*8–12*). BDNF also shows an immediate effect of enhancing synaptic transmission by enhancing neurotransmitter release, possibly through the phosphorylation of presynaptic proteins (*13–17*).

NGF, NT-3, and NT-4/5 and their receptors are also expressed in the central and peripheral nervous systems, and are involved in synaptic plasticity in some subsets of neurons (*4–7*). The receptors for NGF, TrkA, and NT-3, TrkC, are homologous to those of BDNF and NT-4/5, TrkB, however, recent studies suggest that there are several differences in the signal transduction mechanisms among the different neurotrophin receptors (*18*). The application of

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Abbreviations: ACh, acetylcholine; BDNF, brain-derived neurotrophic factor; $[Ca^{2+}]_{in}$, intracellular free $Ca^{2+}$ concentration; DA, dopamine; DMSO, dimethyl sulfoxide; hGH, human growth hormone; $IP_3$, inositol 1,4,5-trisphosphate; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; NGF, nerve growth factor; NT, neurotrophin; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; PLC, phospholipase C.

BDNF elicits a rapid, marked increase in the frequency of spontaneous synaptic currents at synapses in cultured rat hippocampal neurons (*19*), and at neuromuscular synapses in *Xenopus* nerve-muscle co-culture (*20*), where $Ca^{2+}$ influx is essential for the acute effect. The application of NT-3 also induces a marked increase in the frequency of spontaneous synaptic currents in neuromuscular synapses; however, this effect is not dependent on $Ca^{2+}$ influx from extracellular sources, but rather on $Ca^{2+}$ released from intracellular stores through inositol 1,4,5-trisphosphate ($IP_3$) receptors (*21, 22*). The BDNF-induced enhancement of high-frequency transmission at CA1 synapses in the hippocampus appears to be mediated through the activation of the mitogen-activated protein kinase (MAP kinase) and phosphatidylinositol 3-kinase (PI 3-kinase) pathways, but not the phospholipase Cγ (PLCγ) pathway (*23*). In contrast, a concomitant activation of the PI 3-kinase and PLCγ pathways is necessary and sufficient to mediate the NT3-induced synaptic potentiation at *Xenopus* neuromuscular synapses (*22*). NGF/TrkA stimulates the survival of sympathetic neurons primarily by the PI 3-kinase (*18*), whereas the PI 3-kinase and MAP kinase pathways are required for BDNF/TrkB-mediated neuronal survival (*24*). Both BDNF and NT-3 attract growth cone turning of *Xenopus* motoneurons, but the BDNF effect requires $Ca^{2+}$ influx and is dependent on $[cAMP]_{in}$, whereas the NT-3 effect is regulated by $[cGMP]_{in}$ and independent of extracellular $Ca^{2+}$ (*25, 26*).

The signal transduction mechanisms that modulate neurotransmitter release have been studied extensively for BDNF and NT-3 so far, however, few studies have been conducted on NGF. In this study, we examined the effect of NGF on neurotransmitter release from clonal rat pheochromocytoma PC12-C3 cells. We found that NGF enhances neurotransmitter release from PC12-C3 cells through the activation of the MAP kinase and PI 3-kinase pathways without changing in the intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_{in}$). We also found that NGF enhances neurotransmitter release by increasing the number of secretory vesicles in a readily releasable pool.

## MATERIALS AND METHODS

*Materials*—NGF (2.5S form) was prepared from male mouse salivary glands by the method of Suda *et al.* (1978). Polyvinylidene difluoride membranes were from Millipore (Bedford, MA); Dulbecco's modified Eagle's medium and horse serum were from Life Technologies (Rockville, MD); precolostrum new born calf serum was from Mitsubishi Chemicals (Tokyo); ionomycin and U73122 were from Nakarai Tesque (Kyoto); digitonin, K252a, wortomannin, and PD98059 were from Calbiochem (La Jolla, CA); BDNF and U0126 were from Promega (Madison, WI); the monoclonal anti–phosphotyrosine antibody (4G10) was from Upstate Biotechnology (Lake Placid, NY); anti–phosphoprotein kinase B (PKB) and anti–phospho-MAP kinase antibodies were from New England BioLabs (Beverly, MA).

*Cell Culture*—The PC12-C3 cells (*27*), which store large amounts of ACh and DA, were used throughout the present study. They were maintained in Dulbecco's modified Eagle's medium containing 50 ml/liter precolostrum new born calf serum and 50 ml/liter heat-inactivated horse serum. Two days before experiments, $10^6$ cells were plated on polyethyleneimine-coated 35 mm plastic culture dishes.

*Dopamine (DA) and Acetylcholine (ACh) Release Assay*—Before experiments, PC12-C3 cells were washed three times with a low-$K^+$ solution (140 mM NaCl, 4.7 mM KCl, 1.2 mM $KH_2PO_4$, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15 mM HEPES-NaOH, pH 7.4). After pretreating the cells under various conditions as indicated in the figure legends, the cells were incubated for 1 or 2 min in the low-$K^+$ solution and then incubated several times for 1 or 2 min each with either 1 μM ionomycin in the low-$K^+$ solution or a high-$K^+$ solution (95 mM NaCl, 50 mM KCl, 1.2 mM $KH_2PO_4$, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, 15 mM HEPES-Tris, pH 7.4). In some experiments, hypertonic sucrose solution (0.75 M sucrose, 140 mM NaCl, 4.7 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15 mM HEPES-NaOH, pH 7.4) was used to induce DA release. To assay DA and ACh, the solution was immediately transferred to a microtube containing 150 μl of 1M perchloric acid at the end of each incubation period. At the end of the experiments, cells were sonicated on ice with 500 μl chilled 0.2 M perchloric acid and 0.1 mM EDTA. The samples were centrifuged at 15,000 rpm for 5 min at 4°C, and used to measure DA and ACh. The amounts of DA and ACh in the samples were measured as described previously (*27*).

*Secretion of DA from Permeabilized PC12-C3 Cells*—Cell permeabilization was performed at 37°C by the method described originally by Peppers and Holz (*28*) with slight modifications (*27*). The culture medium was removed and the cells were preincubated with 100 ng/ml NGF for 15 min, then rinsed twice with 1 ml of a $Ca^{2+}$-free Locke's solution (5 mM Hepes-NaOH buffer, pH 6.8 containing 156 mM NaCl, 5.6 mM KCl, 0.2 mM EGTA, 3.6 mM $NaHCO_3$, and 5.6 mM glucose). The cells were permeabilized by incubation for 5 min with 250 μl of 8 μM digitonin in KGEP (20 mM Pipes-NaOH buffer, pH 6.8, containing 140 mM potassium glutamate, 5 mM glucose, and 5 mM EGTA). After permeabilization, the medium was replaced with 250 μl of KGEP with or without 100 μM $CaCl_2$. At the end of the incubation periods, the buffer was immediately transferred to a micro tube containing 25 μl of 2 M perchloric acid. Cells were sonicated on ice with 500 μl of chilled 0.2 M perchloric acid. The samples were centrifuged at 15,000 $\times g_{av}$ for 5 min at 4°C, and the supernatant was stored at –80°C until DA assay.

*Transfection*—A DNA fragment corresponding to the human growth hormone (hGH) gene was isolated from pXGH5 vector (Nichols Institute, San Juan Capistrano, CA) and subcloned into pSI vector (Promega, Madison, WI). The resulting vector was named pSIhGH vector. The TrkB cDNA was prepared as described (*29*), and subcloned into pcDNA3 to construct the mammalian expression vector pcDNA3/TrkB. PC12-C3 cells were plated in polyethyleneimine-coated 35-mm culture dishes at a density of $1–2 \times 10^6$ cells/dish. After 18–24 h, the cells were transfected with various vectors using LipofectAMINE (Life Technologies) according to the instruction manual. In the cotransfection experiments, 2.0 μg of pcDNA3/TrkB or empty pcDNA3 vector (as a control) was added with pcDNA3/hGH. The cells were subsequently maintained for 24–48 h and used for hGH release assay. hGH was measured using a radioimmunoassay kit (Nichols Institute) as described (*30*).

*Immunoblotting*—Ten micrograms of cellular protein was separated by SDS-PAGE on linear 4–20% acrylamide

gradient gels. Immunoblotting was performed as described (*31*) using enhanced chemiluminescence (Amersham Pharmacia Biotech, Uppsala, Sweden) and X-ray films for the detection of the immunoreactive signal. Primary antibodies were used at a dilution of 1:1,000.

*$[Ca^{2+}]_{in}$ Measurements*—$[Ca^{2+}]_{in}$ was determined in fura-2 (*32*) loaded cells. After three washes with the low-$K^+$ solution, cells were incubated for 60 min at 37°C in the low-$K^+$ solution supplemented with fura-2/AM at a final concentration of 5 μM. After another 30-min incubation in fura-2/AM-free medium, the cells were examined under a fluorescent microscope equipped under a silicon-intensified-target video camera and a computer-operated imaging unit (IX70, Olympus, Tokyo; Argus-50, Hamamatsu Photonics, Hamamatsu). A pair of fluorescent images was acquired at excitation wavelengths of 340 and 380 nm every 10 s; $[Ca^{2+}]_{in}$ is given as the ratio between the 340 and 380 nm excited fluorescences.

## RESULTS

*NGF Rapidly Enhances $Ca^{2+}$-Dependent DA and ACh Release from PC12-C3 Cells*—In PC12 cells, DA and ACh are stored in large dense-core vesicles and small synaptic microvesicles, respectively, and are released by $Ca^{2+}$-dependent exocytosis from these vesicles (*33*). As shown in Fig. 1, A and B, high-$K^+$–depolarization induced an enormous release of DA and ACh from PC12-C3 cells. When the cells were pretreated with 100 ng/ml NGF for 10 min, the depolarization-induced releases of both DA and ACh were markedly enhanced by 40–50%. The basal releases in low-$K^+$-solution did not change after NGF treatment. Ionomycin is a $Ca^{2+}$ ionophore that induces $Ca^{2+}$-dependent neurotransmitter release without activating voltage-dependent Ca channels. As shown in Fig. 1C, NGF also enhanced ionomycin-induced DA release from PC12-C3 cells, suggesting that NGF enhances DA release by modulating some step after $Ca^{2+}$ influx. A significant stimulant effect of NGF was observed at 5 ng/ml, and the maximum effect was obtained at around 50 ng/ml (Fig. 2A). The enhancement appeared very quickly after a brief lag period and reached a plateau 10–15 min after application (Fig. 2B).

*NGF Enhances Neurotransmitter Release by Increasing the Number of $Ca^{2+}$-Responsive Secretory Vesicles*—Hypertonic solution induces neurotransmitter release from neurons and PC12 cells in the absence of extracellular $Ca^{2+}$ (*34, 35*). As shown in Fig. 1C, NGF was found to potentiate both depolarization and hypertonic solution-induced DA release to a similar extent. Since the hypertonic solution is believed to induce exocytosis of synaptic vesicles in a readily releasable pool in neurons (*34*), these findings suggest that NGF enhances neurotransmitter release by increasing the number of secretory vesicles in the readily releasable pool. To verify this possibility, we examined the effect of NGF on DA release from digitonin-permeabilized PC12-C3 cells. It is well established that $Ca^{2+}$ triggers the exocytosis of secretory vesicles in a readily releasable pool from digitonin-permeabilized PC12-C3 cells in the absence of MgATP (*36*). As shown in Fig. 3, $Ca^{2+}$-induced DA release ceased several min after $Ca^{2+}$ challenge in the absence of MgATP. NGF-treatment prior to membrane permeabilization resulted in an increase in the amount of DA released in the absence of MgATP, suggesting that NGF enhances neurotransmitter

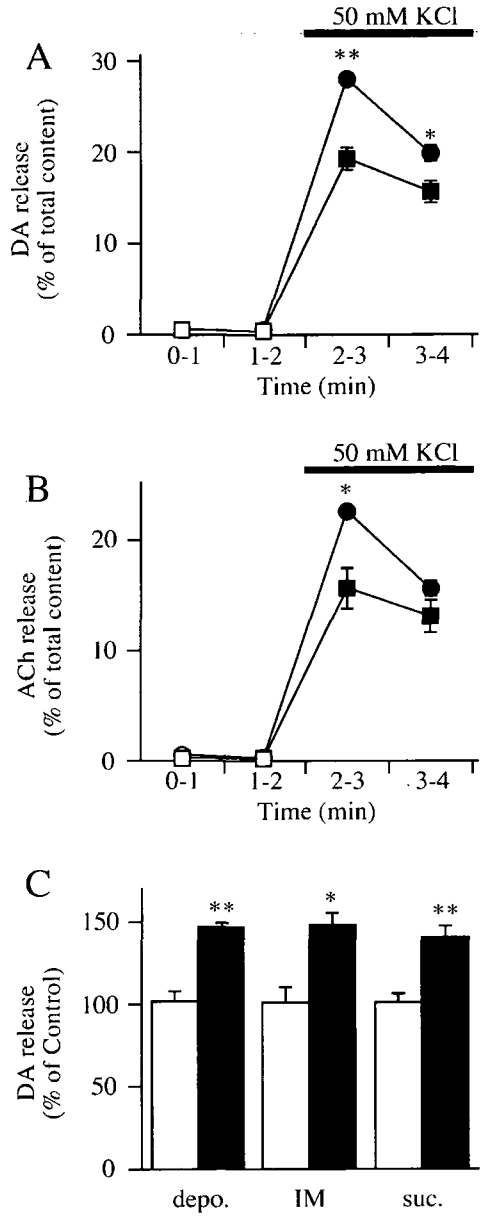


Fig. 1. **The enhancing effects of NGF on DA and ACh release from PC12-C3 cells.** (A) and (B) PC12-C3 cells were incubated in low-$K^+$ solution for 10 min in the presence (circles) or absence (squares) of 100 ng/mL NGF. After treatment, the extracellular solution was replaced every 1 min with the low-$K^+$ solution in the presence or absence of NGF (open circles and squares, respectively). The $K^+$ concentration was elevated from 5 to 50 mM to depolarize the cells in the third and fourth solutions as indicated in the figures in the presence or absence of NGF (filled circles and squares, respectively). The amounts of DA and ACh released into the solution were determined and expressed as a percentage of the total cellular contents. (C) PC12-C3 cells were incubated for 10 min in the presence (filled bars) or absence (open bars) of 100 ng/ml NGF. The extracellular solution was replaced twice at one-minute intervals with the low-$K^+$ solution in the presence or absence of NGF, and the cells were challenged with the following solutions to induce DA release in the presence or absence of NGF (filled and open bars, respectively); depo, the high-$K^+$ solution; IM, 1 μM ionomycin in the low-$K^+$ solution; suc, the hypertonic solution in the absence of $Ca^{2+}$. The relative amounts of DA released in the presence of NGF are expressed as a percentage of the amount released in the absence of NGF. All the values are the means ± SD from three representative experiments (**$p < 0.005$, *$p < 0.01$; Student's *t*-test).

release by increasing the size of the $Ca^{2+}$-responsive vesicle pool in PC12-C3 cells.

*NGF Enhances Neurotransmitter Release through TrkA Activation*—PC12-C3 cells express two types of NGF receptor, a receptor-type tyrosine kinase, TrkA, and the p75 protein (*37*). To examine whether the activation of TrkA receptors is necessary for the NGF-induced enhancement of neurotransmitter release from PC12-C3 cells, we examined the effects of K252a, a potent inhibitor of TrkA tyrosine kinase (*38*), on NGF action. As shown in Fig. 4A, K252a did not have a significant effect on ionomycin-induced DA release, however, it abolished the NGF-induced potentiation of DA release from PC12-C3 cells, indicating that the activation of TrkA is essential for NGF action. PC12-C3 cells do not express TrkB, a high affinity receptor for BDNF, and BDNF did not enhance ionomycin-induced DA release from PC12-C3 cells (Fig. 4B). However, BDNF enhanced the release of hGH, an exogenously expressed reporter molecule of exocytosis of DA-containing large dense-core vesicles (*39*), only from TrkB-expressing cells. Since BDNF activated p75, these findings suggest that p75 does not play a major role in the NGF-mediated enhancement of neurotransmitter release from PC12-C3 cells.

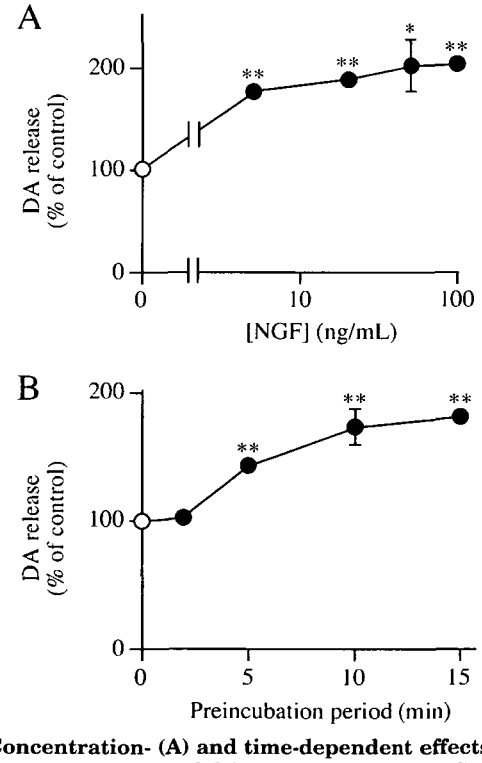


Fig. 2. **Concentration- (A) and time-dependent effects of NGF (B) on ionomycin-induced DA release from PC12-C3 cells.** (A) PC12-C3 cells were preincubated in the low-$K^+$ solution in the presence (filled circles) or absence (open circle) of various concentrations of NGF for 10 min. (B) PC12-C3 cells were incubated in the presence (filled circles) or absence (open circle) of 100 ng/ml NGF for various periods as indicated. The extracellular solution was replaced four times at two-minute intervals with the low-$K^+$ solution, and the cells were challenged with 1 μM ionomycin in the low-$K^+$ solution for 2 min. The amount of DA released in the presence of ionomycin was determined and expressed as a percentage of the amount released without NGF-treatment. All the values are the means ± SD from three representative experiments (**$p < 0.005$, *$p < 0.01$; Student's $t$-test).

*PI 3-Kinase Is Essential for the NGF-Mediated Enhancement of Neurotransmitter Release from PC12-C3 Cells*—Several signaling enzymes, including PI 3-kinase, MAP kinase, and PLCγ, are activated as downstream targets of the TrkA receptor. Wortmannin (*40*) is a potent inhibitor of PI 3-kinase and completely abolished both the NGF-induced potentiation of DA release (Fig. 5A) and the phosphorylation of PKB, a downstream target of PI 3-kinase (Fig. 5B). In contrast, wortmannin did not significantly affect ionomycin-induced DA release in the absence of NGF. These findings thus indicate that PI 3-kinase activation is necessary for the enhancing effect of NGF on neurotransmitter release from PC12-C3 cells.

*MAP Kinase Is Also Essential for the NGF-Mediated Enhancement of Neurotransmitter Release from PC12-C3 Cells*—Next, we examined whether the activation of MAP kinase is necessary for the immediate effect of NGF on neurotransmitter release from PC12-C3 cells. As shown in Fig. 6, two types of MAP kinase kinase (MEK) inhibitors, U0126 (*41*) and PD98059 (*42*), suppressed the NGF-dependent potentiation of DA release and phosphorylation of MAP kinase without any significant effect on the $Ca^{2+}$-dependent release of DA. These findings indicate that the activation of the MAP kinase pathway is also essential for NGF action.

*Activation of PLCγ Is Not Required for the NGF-Dependent Potentiation of Neurotransmitter Release from PC12-C3 Cells*—Finally, we examined the effect of U73122 (*43*), a potent inhibitor of PLCγ, on DA release from PC12-C3 cells. However, it was not possible to assess the involvement of PLCγ from the results of this experiment since U73122 suppressed $Ca^{2+}$-dependent DA release enormously regardless of whether or not the cells were pretreated with

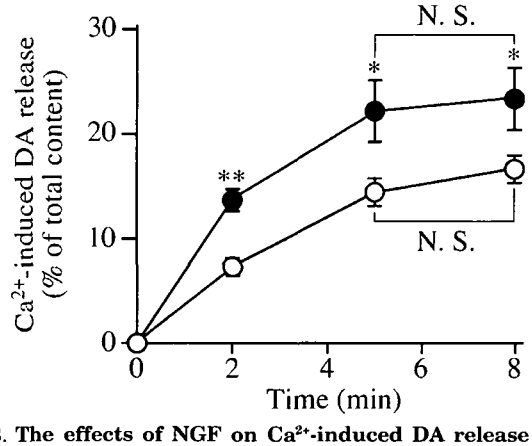


Fig. 3. **The effects of NGF on $Ca^{2+}$-induced DA release from permeabilized PC12-C3 cells.** PC12-C3 cells were incubated for 10 min in the presence (filled circles) or absence (open circles) of 100 ng/ml NGF, and then treated with 10 μM digitonin solution for 5 min to permeabilize the cell membranes. The permeabilized PC12-C3 cells were incubated in releasing medium with or without 100 μM $Ca^{2+}$ for various periods as indicated. The amount of DA released into the medium was determined and expressed as a percentage of total cellular content. $Ca^{2+}$-induced release was calculated by subtracting the release in the absence of $Ca^{2+}$ from that in the presence of $Ca^{2+}$. All values are the means ± SD from three representative experiments (**$p < 0.005$, *$p < 0.01$; Student's $t$-test). No significant differences were observed (Student's $t$-test) between the release at 5 min and 8 min (N. S.).

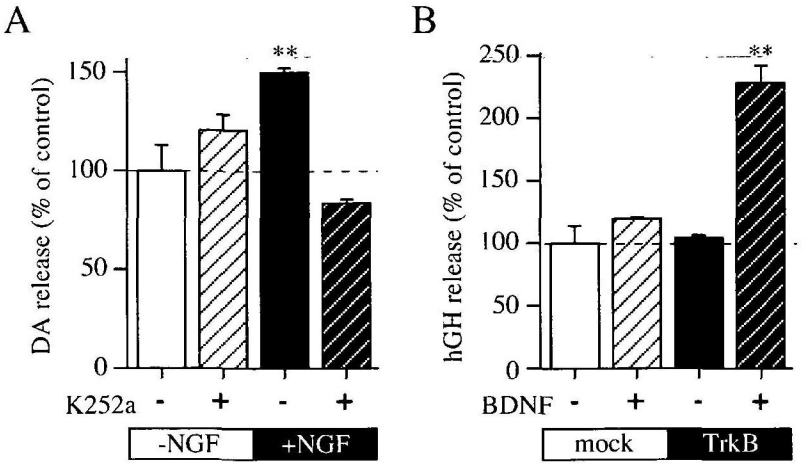


Fig. 4. **Trk receptor-mediated potentiation of ionomycin-induced DA release from PC12-C3 cells.** (A) Effect of K252a on ionomycin-induced DA in the presence (filled bars) or absence (open bars) of NGF-pretreatment. PC12-C3 cells were incubated in the low-$K^+$-solution supplemented with either 50 nM K252a (hatched bars) or 1 ml/liter DMSO (non-hatched bars) for 15 min, followed by a further 15-min incubation in the same solution in the presence (filled bars) or absence (open bars) of 100 ng/ml NGF. Ionomycin-induced DA release over 2 min was measured as described for Fig. 3, and is expressed as a percentage of that released in the absence of NGF and K252a. (B) Effect of BDNF on hGH release from PC12-C3 cells with or without transfection of the TrkB gene. PC12-C3 cells were transiently transfected with the hGH gene with (filled bars) or without (open bars) the TrkB gene. Two days after transfection, the cells were incubated in the low-$K^+$-solution for 10 min with (hatched bars) or without (non-hatched) 50 ng/ml BDNF. After washing with the low-$K^+$-solution, the cells were incubated in 1 μM ionomycin-containing low-$K^+$-solution for 2 min, and the amount of hGH released into solution was determined and expressed as a percentage of that released in the absence of BDNF without TrkB transfection. All values are the means ± SD from three representative experiments (**$p < 0.005$; Student's $t$-test).

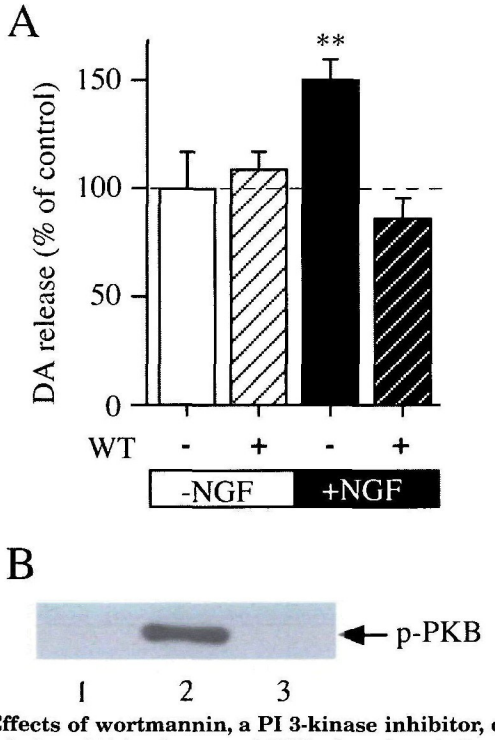


Fig. 5. **Effects of wortmannin, a PI 3-kinase inhibitor, on ionomycin-induced DA release and PKB phosphorylation in the presence or absence of NGF.** (A) PC12-C3 cells were incubated in the low-$K^+$-solution supplemented with either 50 nM wortmannin (hatched bars) or 1 ml/liter DMSO (non-hatched) for 15 min, followed by a 10-min incubation in the same solution in the presence (filled bars) or absence (open bars) of 100 ng/ml NGF. Ionomycin-induced DA release over 2 min was measured as described for Fig. 3, and is expressed as a percentage of that released in the absence of NGF and wortmannin. All values are the means ± SD from three representative experiments (**$p < 0.005$; Student's $t$-test). (B) The phosphorylation of PKB, a downstream enzyme of PI 3-kinase, was examined in homogenates of PC12-C3 cells by immunoblotting probed with anti–phospho-PKB antibody. 1, control cells; 2, cells treated with NGF; 3, cells treated with wortmannin and NGF.

NGF (Fig. 7A). The activation of PLCγ generates $IP_3$, which induces an elevation of $[Ca^{2+}]_{in}$ through the activation of $IP_3$ receptors in intracellular $Ca^{2+}$ store sites. Bradykinin receptors are coupled with PCLβ, and $IP_3$ is also generated after receptor activation. As shown in Fig. 7B, bradykinin, but not NGF, induced an elevation of $[Ca^{2+}]_{in}$ in PC12-C3 cells, suggesting that the production of $IP_3$ sufficient to elevate $[Ca^{2+}]_{in}$ was not induced by NGF in PC12-C3 cells.

## DISCUSSION

In this study, we found that NGF induces a rapid enhancement of ACh and DA release from PC12-C3 cells. We also showed that NGF increases the number of secretory vesicles in a readily releasable pool, and both PI 3-kinase inhibitor and MEK inhibitor suppress the action of NGF.

Previously, NGF was reported to induce $Ca^{2+}$ influx into PC12 cells and subsequent DA release (*44, 45*). PC12 cells become heterogeneous after a large number of cell divisions due to spontaneously generated mutations, and many laboratories including ours have conducted subcloning of PC12 cells using the intrinsic heterogeneity of the cells to isolate lines with particular phenotypes (see Ref. *46*). We found that NGF-induced $Ca^{2+}$ mobilization differs among different subclones, and NGF induces a transient increase in $[Ca^{2+}]_{in}$ in some subclones but not in others, including the PC12-C3 cells used in these studies (see Fig. 7B). In contrast, NGF-induced enhancement of neurotransmitter release was observed in all subclones tested. We also showed here that NGF-enhanced neurotransmitter release is evoked not only by high-$K^+$-depolarization, but also by ionomycin treatment. These findings suggest that NGF enhances $Ca^{2+}$-dependent neurotransmitter release by modulating step(s) subsequent to $Ca^{2+}$ entry.

We found that NGF enhances the $Ca^{2+}$-dependent release of both ACh and DA, which are stored in small synaptic microvesicles and large dense-core vesicles, respectively (*33*). Protein composition, mechanism of biosynthesis and recycling, and the requirement for $Ca^{2+}$-sensing proteins differ between these two types of vesicles. On the other

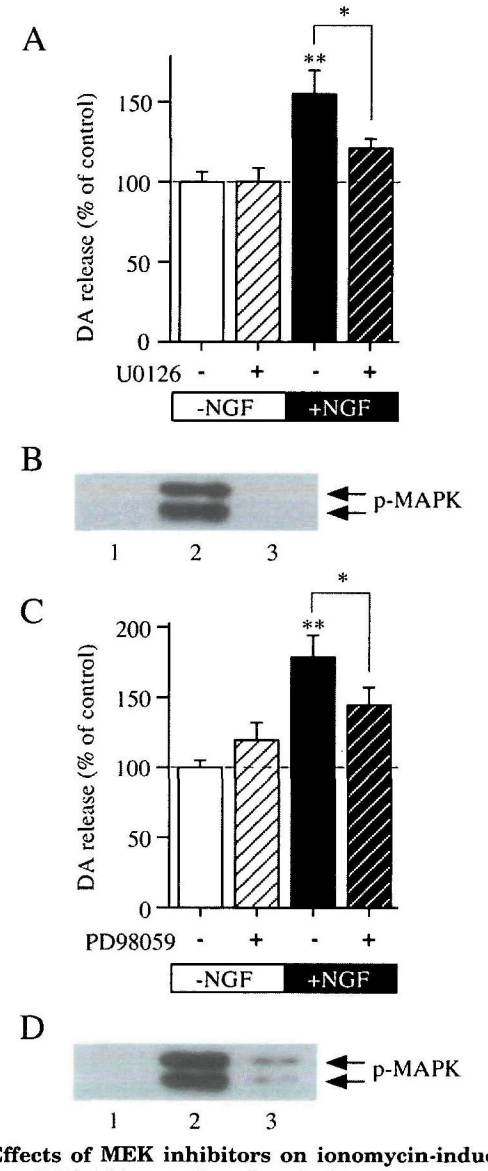


Fig. 6. **Effects of MEK inhibitors on ionomycin-induced DA release and MAP kinase phosphorylation in the presence or absence of NGF.** (A) and (C), PC12-C3 cells were incubated in the low-$K^+$-solution supplemented with either inhibitor [1 μM U0126 for (A) or 50 μM PD98059 for (C); hatched bars] or 1 ml/liter DMSO (non-hatched) for 15 min, followed by a 10-min incubation in the same solution in the presence (filled bars) or absence (open bars) of 100 ng/ml NGF. Ionomycin-induced DA release over 1 min was measured as described for Fig. 3, and is expressed as a percentage of that released in the absence of NGF and inhibitor. All values are the means ± SD from three representative experiments (**$p < 0.005$, *$p < 0.01$; Student's $t$-test). (B) and (D) The phosphorylation of MAP kinase was examined in homogenates of PC12-C3 cells by immunoblotting probed with anti–phospho-MAP kinase antibody. 1, control cells; 2, cells treated with NGF; 3, cells treated with NGF and either U0126 (B) or PD98059 (D).

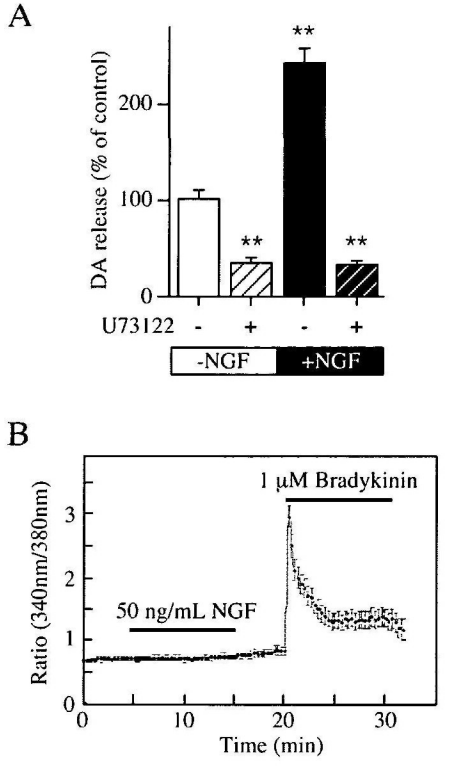


Fig. 7. **Evaluation of the involvement of PLCγ in the action of NGF in PC12-C3 cells.** (A) Effects of U73122, a PLCγ inhibitor, on ionomycin-induced DA release from PC12-C3 cells in the presence or absence of NGF. PC12-C3 cells were incubated in the low-$K^+$-solution supplemented with either 10 μM U73122 (hatched bars) or 1 ml/liter DMSO (non-hatched) for 15 min, followed by a 10-min incubation in the same solution in the presence (filled bars) or absence (open bars) of 100 ng/ml NGF. Ionomycin-induced DA release over 2 min was measured as described for Fig. 3, and is expressed as a percentage of that released in the absence of NGF and U73122. All values are the means ± SD from three representative experiments (**$p < 0.005$; Student's $t$-test). (B) The effect of NGF and bradykinin on $[Ca^{2+}]_{in}$ in PC12-C3 cells. $[Ca^{2+}]_{in}$ was measured by microfluorometry in fura-2–loaded cells. The fluorescence was measured every 10 s as described in "MATERIALS AND METHODS," and $[Ca^{2+}]_{in}$ is expressed as a ratio of emission intensities (340 nm/380 nm). NGF (50 ng/ml) and 1 μM bradykinin were added successively to the low-$K^+$ solution as indicated by the horizontal bars. The values represent means ± SE ($n = 7$).

hand, they have many common properties, including ATP-dependent priming, GTP-sensitivity, and phorbol ester–mediated regulation of exocytosis (*27*). These findings indicate that exocytosis of these two types of vesicles can be modulated equally by NGF, possibly through the post-translational modification of protein machinery common to both types of vesicles.

Recent studies have shown that there are many different steps that occur prior to the fusion of secretory vesicles to the plasma membrane, and all of these steps can be modulated by various protein kinases (*47*). Hypertonic solutions induce neurotransmitter release from neurons and PC12 cells in the absence of extracellular $Ca^{2+}$ (*34, 35*). In neurons, hypertonic solutions are believed to induce the exocytosis of synaptic vesicles in a readily releasable pool (*34*). We show here that NGF also enhances neurotransmitter release evoked by a hypertonic solution from PC12-C3 cells, suggesting that NGF treatment causes an increase in the number of secretory vesicles in a readily releasable pool. This suggestion is further supported by the experiment using digitonin-permeabilized leaky cells. In the absence of ATP, $Ca^{2+}$-induced DA and ACh releases ended quickly because the ATP-dependent recruitment of $Ca^{2+}$-responsive

vesicles was not available (*36*). In other words, the amount of $Ca^{2+}$-induced release in the absence of ATP reflects the number of vesicles responsive to $Ca^{2+}$. We found that NGF treatment increased the amount of DA release from leaky cells in the absence of ATP by 40–50% (Fig. 3). All of these findings indicate that the number of $Ca^{2+}$-responsive vesicles is increased by NGF-treatment. In preliminary experiments, we transiently expressed green fluorescence protein–conjugated vesicular monoamine transporter and vesicular ACh transporter to visualize large dense-core vesicles and small synaptic microvesicles, respectively. The distributions of these vesicles were markedly changed by phorbol ester but not by NGF. These results suggest that NGF enhances neurotransmitter release by modulating some step(s) after docking, but further work is needed to clarify this issue.

Two types of NGF receptors, TrkA and p75, are expressed in PC12-C3 cells. K252a, a potent inhibitor of Trk tyrosine kinases, abolishes the NGF-induced potentiation of DA release without any significant effect on $Ca^{2+}$-evoked DA release. NGF, as well as BDNF, activates P75, however, BDNF has no enhancing effect on $Ca^{2+}$-dependent neurotransmitter release from PC12-C3 cells unless the TrkB receptor gene is transfected into the cells. All of these findings clearly indicate that TrkA tyrosine kinase is essential for the NGF-mediated regulation of neurotransmitter release from PC12-C3 cells, whereas p75 by itself is not able to stimulate neurotransmitter release from PC12-C3 cells. However, we can not exclude the possibility that p75 plays a supportive role for TrkA receptors.

The activation of Trk receptors leads to various phenomena in different time scales (*4–7*). The stimulant action of NGF on $Ca^{2+}$-dependent neurotransmitter release occurs within 15 min after application, suggesting that it depends not on transcription and/or translation, but on post-translational modification, possibly the phosphorylation of preexisting proteins. NGF treatment induces the activation of PI 3-kinase and MAP kinase, and the phosphorylation of PKB and MAP kinase was observed in PC12-C3 cells. Specific inhibitors of PI 3-kinase and MAP kinase abolish the NGF-induced potentiation of neurotransmitter release without significant effects on the release. This indicates that the activation of both PI 3-kinase and MAP kinase is essential for the NGF-mediated potentiation of neurotransmitter release from PC12-C3 cells. Src kinase and PLCγ act downstream of TrkA receptors (*37*). However, src-family kinases are not likely to be involved in the regulation since we recently found that they negatively regulate neurotransmitter release from PC12 cells and cultured cerebellar granule cells (*48*). We could not examine the possible involvement of PLCγ in the regulation using U73122, a potent inhibitor of PLCγ, since it inhibited $Ca^{2+}$-induced release itself. The activation of PLCγ generates $IP_3$, which induces $Ca^{2+}$ release from intracellular $Ca^{2+}$ storage sites through the activation of $IP_3$ receptors. Bradykinin induces a transient increase in $[Ca^{2+}]_{in}$ in PC12-C3 cells through the activation of PLCβ-coupled receptors, however, NGF does not induce any significant change in $[Ca^{2+}]_{in}$. These findings may suggest that PLCγ is not involved in the NGF-mediated enhancement of neurotransmitter release from PC12-C3 cells. In summary, the mechanism of NGF action is different from the mechanisms of BDNF and NT-3 action in its requirement for $[Ca^{2+}]_{in}$ elevation (*19–22*), and from the mechanisms of NT-3 action in its requirement for MAP kinase (*22*).

BDNF plays a predominant role in the central nervous system. However, NGF also plays important roles in the central and peripheral nervous systems during development and in adulthood (*16, 49–55*). Different neurotrophins play different roles in synaptic plasticity even in the same area of the brain. For example, BDNF and NT-3 are involved in long-term and short-term plasticity in rat dentate gyrus, respectively (*56, 57*). It is thus important to elucidate further the physiological roles of NGF-mediated regulation of neurotransmitter release in brain function.

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