Expression of the δ-opioid receptor gene (dor) is tightly controlled during neuronal differentiation and developmental stages. Such distinct temporal and spatial expression of dor during development suggests a role for the δ-opioid receptor in early developmental events. However, little is known about intracellular signaling pathways that control dor expression. A well established cell line model for the study of gene expression during neuronal differentiation is the rat adrenal pheochromocytoma PC12 cell line. Here we found that the constitutively activated TrkA/phosphatidylinositol 3-kinase/Akt (protein kinase B)/NF-κB survival cascade mediates dor expression during nerve growth factor (NGF)-induced differentiation of PC12h cells. Biochemical experiments showed that constitutive phosphorylation of Akt and IκBα correlates with NGF-induced dor expression. Overexpression of the transcriptional activator NF-κB/p65 increased dor promoter activity. Overexpression of the NF-κB signaling super inhibitor mutant IκBα (S32A/S36A) abolished the effect of p65 and blocked NGF-induced activation of NF-κB signaling, resulting in a significant reduction in dor promoter activity. Treatment with SN50, an NF-κB-specific nuclear translocation peptide inhibitor, inhibited the translocation of NF-κB, resulting in a reduction of dor mRNA. The gel shift assay supported the fact that there exists an NF-κB-binding site on the dor promoter. RNA interference experiments using NF-κB/p65 small interfering RNA confirmed that NF-κB signaling is required for dor expression. Our findings not only provide a new mechanistic explanation for NGF-induced dor expression but also shed some light on the molecular mechanism of the temporal and spatial expression of dor and the roles of the δ-opioid receptor during neuronal differentiation.

The opioid receptors belong to the rhodopsin family of the G protein-coupled receptor superfamily. The opioid receptor subfamily is composed of three subsets of the gene products that include the κ-, δ (DOR), and η-opioid receptors and plays an important role in the pain control mechanism. The δ-opioid receptor gene (dor) is temporally and spatially expressed in developmental stages (1, 2). Such distinct temporal and spatial patterns of dor expression during development suggest that DOR may have a role in early developmental events (1).

A well established cell line model for the study of gene expression during neuronal development is the rat adrenal pheochromocytoma PC12 cell line (3). Nerve growth factor (NGF) could induce 10% of the total genes in PC12 cells (4). NGF can significantly increase binding of the DOR agonist Met-enkephalin to the surface of PC12h (a PC12 subclone) cells in a time-dependent manner (5). Further studies indicated that NGF could enhance the dor mRNA level in PC12h cells (6). Despite the discovery of NGF induction of dor expression more than two decades ago, little is known about the intracellular signaling mechanism that mediates dor expression.

NGF plays a critical role in the development and maintenance of the central nervous system and peripheral nervous system (7). During neuronal development, neurotrophins control the expression of many neuronal survival-associated genes, including the nuclear factor κB (NF-κB)-dependent bcl-xL gene (8). Such control is mainly mediated by activation of phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B) signaling, which leads to the phosphorylation of downstream transcription factors, including NF-κB (9, 10). The Dor protein and its peptide agonists promote the survival of neurons in the central nervous system and the preserved peripheral organs (11). Opioid ligands can activate PI3K/Akt survival signaling in the opioid receptor-transfected HEK293 cells (12), suggesting that the opioid survival effects may be attributable to activation of PI3K/Akt signaling. Moreover, the computer data base search indicated a putative NF-κB cis-element on the dor promoter (13). Taken together, we hypothesized that NGF-mediated PI3K/Akt signaling may be involved in the expression of the survival-associated dor gene during neuronal differentiation. As the first step of this investigation, we tested this hypothesis in the NGF-responsive PC12h cell model system.

In this study, using specific signaling inhibitors and biological agents in combination with the small interfering RNA (siRNA) approach, we found that NGF receptor TrkA and the downstream PI3K/Akt signaling cascade, not the mitogen-activated protein kinase (MAPK), phospholipase Cγ, and protein kinase C signaling cascades, serves as the main pathway required for dor expression in the NGF-differentiated PC12h cell model. We further found that sustained activation of Akt and NF-κB is important for dor expression. Thus, this mechanism of NGF-induced dor expression may account in part for the temporal and spatial expression of dor during development.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The PC12h cell line was a generous gift from Dr. Hiroshi Hatanaka. PC12h cells were grown and maintained in 1:1 Ham’s F-12 medium/Dulbecco’s modified Eagle’s medium plus 5% horse serum and 5% calf serum as described elsewhere (5). Nerve
PI3K/Akt/NF-κB Signaling-mediated dor Expression

growth factor (2.5 S) was purchased from Harlan Bioproducts for Science, Inc. (Indianapolis, IN). LY294002, PD98059, K-252a, U-73122, bisindolylmaleimide I, and brain-derived neurotrophic factor (BDNF) were purchased from Calbiochem (La Jolla, CA). Cell-permeable NF-κB inhibitory peptide SN50 and its mutant SN50M were purchased from both Biomol (Plymouth Meeting, PA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-p50 (E-10), anti-p52 (C-5), and anti-p65 (F-6) monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. Total RNA of the rat brain was purchased from Clontech (Palo Alto, CA).

**Plasmid Constructs**—The dor promoter-containing reporter gene constructs pD1300 and pD262 were reported previously (14). Multiple putative transcription initiation sites were located by RNase protection assay in a TATA-less G+C-rich sequence between 390 and 140 nucleotides upstream from the ATG translation start codon (13). The promoterless and enhancerless luciferase vector pGL3-basic was from Promega (Madison, WI). pNFκB-Luc is a reporter vector especially designed for monitoring activation of the NF-κB signaling pathway (Clontech). pNFκB-Luc contains multiple copies of the NF-κB consensus sequence (GGGAATTTCC) fused to a TATA-like promoter region from the Herpes simplex virus thymidine kinase promoter. The pCMV-IκBαM (IKBM) construct is an IκBα dominant-negative vector (Clontech). After IKBM is transfected into cells, expression of super inhibitor IκBα (S23A/S36A) mutant protein (IKBM) results in specifically blocking NF-κB signaling in a particular cell line. Constructs pCMV4 and pCMV4/p65 are generous gifts from Dr. Warner C. Greene (University of California, San Francisco, CA).

**Transfection of PC12h Cells and Reporter Gene Assay**—PC12h cells (8 × 10^5 cells/60-mm dish) were transiently transfected for 3 h with 2.5 pmol of individual DNA constructs and a 1:5 molar ratio of pCH110 cis (Amersham Biosciences) or 0.5 μg of Renilla luciferase expression vector pRL-TK (Promega) using SuperFect or Effectene transfection reagent (Qiagen) unless otherwise noted. The transfection was carried out according to the manufacturer’s protocols. After transfection, the cells were cultured for 48 h in a Ham’s F-12 medium/Dulbecco’s modified Eagle’s medium (1:1) containing 10 μg/ml of insulin, 6.7 ng/ml of sodium, and 5.5 ng/ml of transferrin with or without 50 ng/ml of NGF. The cells were harvested, lysed, and assayed for luciferase activities using a luciferase reporter assay system (Promega) according to the manufacturer’s protocols. The net luciferase activity of each plasmid was obtained by subtraction of the activity of the empty vector pGL3-basic from the total activity of each plasmid. The differences in transfection efficiency from dish to dish were corrected by using Renilla luciferase or β-galactosidase activities of each lysate sample. In addition, the protein concentration of each sample was determined by using a Pierce BCA protein assay kit. The relative luciferase activity was normalized to correct the difference in the amount of protein used for each assay.

**Western Blot Analysis of Phosphorylation of Akt and IκBα**—The proteins from cell lysates were separated on 10% SDS-PAGE gels and blotted onto nitrocellular membranes according to the manufacturer’s protocols (Cell Signaling, Beverly, MA). Phosphorylated Akt and IκBα as well as unphosphorylated Akt and IκBα on the membranes were then probed by anti-p-Akt (Ser^473), anti-p-IκBα (Ser^32), anti-Akt, and anti-IκBα according to the manufacturer’s protocol (Cell Signaling), respectively. Identification and quantification of protein bands were as described previously (15) unless otherwise noted.

**Reverse Transcription (RT)-PCR of the Endogenous dor Gene**—Total RNA was isolated from PC12h cells using a TRI Reagent® kit (Molecular Research Center, Cincinnati, OH). RT-PCR was performed on 4 μg of total RNA using a Qiagen One-step RT-PCR kit with specific primers. A primer set specifically for the dor gene was used to produce a 365-bp product as described previously (16). For the dor transcripts, semi-quantitative RT-PCR was carried out by a standard protocol from the Qiagen kit: reverse transcription at 50 °C for 30 min; initial PCR activation at 95 °C for 15 min; 39–43 cycles at 94 °C for 0.5 min, 55 °C for 0.75 min, and 72 °C for 1 min; and finally 72 °C for 10 min. Because the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level did not change with NGF treatment (4), the GAPDH RT-PCR product served as an internal control for normalization. Equal aliquots of PCRs were analyzed on a 2% agarose gel and photographed by a Kodak Digital Science DC120 Zoom digital camera. Semi-quantitative analysis of the RT-PCR products was carried out by using ImageQuant software (Amersham Biosciences). The dor-specific signal was normalized to the GAPDH-specific signal in each reaction to obtain a value of relative fluorescence intensity.

**Electrophoretic Mobility Shift Assay**—The recombinant transcription factor NF-κB (p50) protein and activating protein-1, NF-κB, and octamer transcription factor 1 consensus double-stranded oligonucleotides were purchased from Promega. Single-stranded oligonucleotides were synthesized by Integrated DNA Technologies, Inc. The probe used was a double-stranded oligonucleotide DorsNFκB containing a putative NF-κB cis-element on the dor promoter. The probe was labeled at the 5’-end with ^32P by the T4 DNA polynucleotide kinase using the labeling kit from Promega. The probe and other oligonucleotide sequences are listed in Table 1. Cells treated with NGF for different periods of time were harvested, and cytosolic proteins and nuclear proteins were extracted according to the modified protocol of Dignam and Roeder (17). The protein concentrations were determined by using the Pierce BCA protein assay kit. For each binding reaction, 70 fmol of the ^32P-labeled probe (50,000–90,000 cpm) were incubated with 0.4 μg gel shift unit of NF-κB p50 protein or 10 μg of PC12h cytosolic or nuclear protein extracts at room temperature for 20 min (a gel shift unit was defined by Promega as the amount of p50 required to shift 0.38 pmol of the NF-κB oligonucleotide under the manufacturer’s conditions). The binding buffer contained 4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl, and 0.05 mg/ml of poly(dI-dC)-poly(dI-dC) for nuclear extracts. When the recombinant NF-κB p50 was used, the binding buffer was 10 mM HEPES (pH 7.9) containing 50 mM KCl, 0.1 mM EDTA, 2.5 mM dithiothreitol, 10% glycerol, and 0.05% Nonidet P-40. For the immunoshift experiment, 4 μg of mouse monoclonal antibodies (anti-p50, anti-p52, and anti-p65) against the NF-κB subunits p50, p52, and p65 were added, respectively, to the reaction for an additional 20 min.

**RNA Interference**—PC12h cells in 60-mm dishes were transfected with NF-κB/p65 siRNAs (siRNA SMARTpool™ p65, M-Q-080033-00, and SMARTselected duplexes, M-Q-080033-00-0005; Dharmacon, Lafayette, CO) following the Lipofectamine 2000 protocol from the manufacturer (Invitrogen). A nonspecific siRNA control pool

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**TABLE 1**

| Probe/competitors | Sensitivity |
|-------------------|-------------|
| AP-1              | 5'-GCCCTTTGAGCTCGACCGGAAA (−172 to −151) |
| DorNFκB           | 5'-GCCCTTTGAGCTCGACCGGAAA (−172 to −151) |
| DorNFκBm          | 5'-GCCCTTTGAGCTCGACCGGAAA (−172 to −151) |
| NF-κB             | 5'-AGTTGAGGAGGAGTTCCAGGCCC |
| OCT-1             | 5'-TCTGCAATGACAACTTAGAA |
RESULTS

Time-dependent Induction of dor Expression by NGF in PC12h Cells—To investigate the effect of NGF on the dor mRNA level, PC12h cells were treated with 100 ng/ml of NGF for a time period of 48 h. RT-PCR analysis was carried out using the One-step RT-PCR kit (Qiagen) according to the manufacturer’s protocol under semi-quantitative experimental conditions as shown in Fig. 1 (A and B). NGF induced dor mRNA at about 4 h, and a significant increase in dor mRNA was observed at 24 h (Fig. 1C). At 48 h, NGF treatment of PC12h cells resulted in a 3.8-fold increase in the dor mRNA (Dunnnett’s test, p < 0.001) (Fig. 1D). This level of dor mRNA is comparable with that in the rat brain (Fig. 1D). NGF also induced expression of the dor gene in parental PC12 cells, although with a lower induction fold (data not shown). Thus, in this study only the subclone PC12h cells were used.

Inhibition of TrkA/PI3K Signaling Resulted in Blockage of dor Expression—Both NGF receptors TrkA and p75

NGF (Fig. 2A) and the activity of dor promoter-driven reporter pD1300, which contains the 5′-flanking sequence from −1.3K to +1 of the dor promoter (translational start site designated as +1) (14) (Fig. 2B). Furthermore, if NGF up-regulation of dor promoter activity had been mediated through direct activation of p75

NGF, BDNF treatment might have had similar effect on promoter activity because both NGF and BDNF have similar binding affinities to p75

When PC12 cells were transfected with pD1300 and stimulated with BDNF for 48 h, the BDNF-induced dor promoter activity was not significant in comparison with the control (p > 0.05) (Fig. 2B). The BDNF result indicates that p75

Although the low affinity NGF receptor p75ntr can also directly interact with NGF (20), hence, either of these neurotrophin receptors could be involved in NGF-induced up-regulation of dor mRNA. TrkA is a tyrosine kinase that autophosphorylates itself upon activation by NGF, but p75

NGF treatment resulted in an increase in phosphorylation of Akt at serine 473. The p-Akt peaked at 15–30 min, was slowly dephosphorylated (Fig. 2D), and sustained at a significant level for 48 h (p < 0.05; Fig. 3A and B)
PI3K/Akt/NF-κB Signaling-mediated dor Expression

Effects of IkBa Mutant (S32A/S36A), NF-κB/p65, and Nuclear Translocation Inhibitor SN50 on NGF-induced dor Expression—There are several downstream potential effectors of Akt in a certain cell type. One of them is IkB kinase, the activation of which leads to phosphorylation of IkB (28). LY294002 treatment decreased the p-Akt (Ser473) level and p-IkBα (Ser27/32) level, suggesting that NF-κB signaling may be involved in NGF/PI3K/Akt-dependent dor expression. A previous computer data base search also indicated that a putative NF-κB-binding site was located in the 5′-flanking region (−172 to −151; see Table I for sequence) of the dor promoter (13). To test the involvement of NF-κB signaling in dor expression, we carried out a series of experiments as summarized in Fig. 4. Transfection of the NF-κB transactivating subunit p65-expressing plasmid resulted in a more than 50% increase in the promoter activity of pD262, which is a dor promoter-driven reporter containing the sequence from −262 to +1 of the 5′-flanking region of the dor promoter (14), whereas cotransfection of the NF-κB super inhibitor IκBα M-expressing plasmid (IKBM) into the cells blocked p65 transactivating activity (Fig. 4A).

We then tested whether NGF treatment would activate NF-κB signaling in PC12h cells. As shown in Fig. 4B, NGF induced NF-κB-specific enhancer-driven reporter activity by about 15-fold, and the activation was significantly reduced after cotransfection with IKBM. A parallel result was also obtained for the dor promoter-driven reporter pD262 (Fig. 4B).

To examine whether translocation of the NF-κB active complex to the nucleus is required for NGF-induced dor expression, we evaluated the effect of the cell-permeable NF-κB-specific peptide inhibitor SN50 and its mutant SN50M on the level of NGF-induced dor mRNA. SN50 and SN50M are 24-mer peptides. SN50 contains the NLS of the NF-κB p50 subunit and SN50M contains the NLS with two residues mutated (29). Fig. 4C shows that treatment with 100 μg/ml of SN50 completely abolished the effect of NGF on the regulation of dor mRNA, whereas the mutant SN50M (100 μg/ml) had no effect on it. These results further confirm that NF-κB signaling is involved in dor expression in PC12h cells.

The dor Promoter Contains an NF-κB-binding Site—To test whether the dor promoter has a real NF-κB-binding site, we carried out EMSA using

FIGURE 3. Western blot analysis of total and phosphorylated protein levels of Akt and IkBα in PC12h cells. A, NGF-induced phosphorylation of Akt and IkBα. PC12h cells were treated with 100 ng/ml NGF. p-Akt, phosphorylated Akt (Ser473); p-IkBα, phosphorylated IkBα (Ser27/32). NS denotes the nonspecific band used as internal control for protein loading and for normalization of the optical density of each band (see “Experimental Procedures”). B, semi-quantification of p-Akt and p-IkBα. Relative optical density (OD) was measured as described under “Experimental Procedures.” The data are the means ± S.D. (**, p < 0.01; *, p < 0.05; n = 3). C, LY 294002 resulted in a reduction in the sustained p-Akt and p-IkBα levels. LY, LY 294002; PD, PD 98059. PC12h cells were treated with mock control (Ctl, MeSO₃), 10 μM PD 98059, and 20 μM PD 98059 for 48 h. The data were the means ± S.D. (n = 3).

B). We also observed that a transient decrease in the Akt protein level occurred after cells were treated with NGF for 30 min to 4 h (Fig. 3A). Meanwhile, phosphorylation of IkBα at Ser32 also peaked at 15 min, but the rate of dephosphorylation was quicker than that of p-Akt (Fig. 3, A and B). Subsequently, the decrease in IkBα occurred between 30 min and 4 h. Treatment with PI3K inhibitor LY29402 resulted in a significant reduction in p-Akt and its downstream effector p-IkBα at 48 h (p < 0.05; Fig. 3C), indicating that the constitutive phosphorylation of Akt and IkBα may play a critical role in NGF/PI3K signaling-dependent dor expression.

FIGURE 2. NGF induction of dor mRNA and promoter activity via TrkA/PI3K signaling. A, effect of K-252a on the NGF up-regulation of dor mRNA. The result shown in A is a representative of three independent experiments that are reproducible. B, effects of K-252a and BDNF on dor promoter activity. C, effect of PD 98059, LY294002, U-73122, and bisindolylmaleimide I on dor promoter activity. PD, PD 98059 (20 μM); LY, LY 294002 (10 μM); U73, U-73122 (1 μM); BIM, bisindolylmaleimide I (1 μM). For both B and C, the transfected cells were harvested, and the relative luciferase activity (RLU) was assayed as described under “Experimental Procedures.” The untreated cell sample was used as a control. The data are the means ± S.E. of two to four independent experiments in duplicate. D, effects of PD98059 and LY294002 on the level of dor mRNA. NGF, 50 ng/ml; LY294002, 10 μM; PD98059, 20 μM, and K-252a as control (50 nM). PC12h cells were treated with 50 nM of the Trk kinase-specific inhibitor K-252a for 48 h. Treatment of PC12 cells with drugs, harvesting total RNA, and RT-PCRs were described under “Experimental Procedures.”
FIGURE 4. Effects of IκBα mutant (S32A/S36A), NF-κB/p65, and nuclear translocation inhibitor SN50 on NGF-induced dor expression. A, effect of overexpression of the trans-activating factor NF-κB p65 on the activity of dor promoter. p65, pCMV/p65 construct; IKBM, pCMV-IκBα, which is a dominant-negative IκBα mutant vector that expresses the mutant protein IκBα (S32A/S36A). Control pCMV vector was used for keeping total DNA as a constant for all transfection experiments. The data are the means ± S.E. of three independent experiments in duplicate (**, p < 0.01). B, effect of overexpression of mutant IκBα on NF-κB activation with the binding result from HeLa cell nuclear extracts (Fig. 5A, lane 3). The protein-DNA complex formation was further blocked by a 50-fold excess of competitor oligonucleotides (Fig. 5A, lane 1) and by a 20- and 50-fold excess of NF-κB consensus sequence oligonucleotides (30) (Fig. 5A, lanes 10 and 11) but could not be blocked by the mutant DorNFkB (DorNFkBmt) and the nonspecific octamer transcription factor 1 (Fig. 5A, lanes 6–9).

Similar results were obtained from the binding reaction between the nuclear extracts from PC12h cells and the labeled probe DorNFkB (Fig. 5B). Adding an excess of unlabeled probe DorNFkB to the binding reaction competed off this putative NF-κB p50/p65 heterodimer complex (Fig. 5B). The presence of nonspecific activating protein-1 and octamer transcription factor 1 consensus oligonucleotides and the mutant DorNFkBmt did not affect the complex formation significantly (Fig. 5B, top panel).

If NGF-induced dor expression is mediated via activation of NF-κB signaling, then one of the consequences of NGF stimulation will be an up-regulation of dor mRNA. PC12h cells were treated with the cell membrane-permeable p50, p52, and p65 were used to block the protein-DNA complex formation. Both anti-p50 and anti-p65 interfered the complex formation, but anti-p52 did not (Fig. 5C, lanes 1–5 in the left panel). This result is also consistent with the binding result from HeLa cell nuclear extracts (Fig. 5C, lanes 6–8 in the left panel); the antibodies destroyed the major part of the protein-DNA complex just as we observed in PC12h cell nuclear extracts (Fig. 5C, lanes 7 and 8 in the left panel). Only a faint supershift band was observed in HeLa nuclear extracts after treatment with anti-p50 and anti-p65 monoclonal antibodies. It is known that HeLa cells have a high level of activated NF-κB proteins in the nucleus (31). Our result also showed that HeLa cells have a much higher level of activated NF-κB proteins than that in PC12h cells (Fig. 5C, left panel). Thus, it is very likely that because of a result of a low amount of NF-κB in PC12h cells, we did not see a clearly anti-p65 antibody-
induced supershift band in PC12h nuclear extracts (Fig. 5C, lanes 4 and 5 in the left panel). To investigate this matter further, we evaluate the antibody treatment with the binding reactions in the presence of one half amount of HeLa nuclear extracts used in lanes 6–8 of Fig. 5C (left panel). Under these conditions we observed the protein-DNA complex band, and the complex formation was specifically blocked in the presence of anti-p50 and p65 antibodies without a clear supershift band (Fig. 5C, lanes 1–3 in the right panel). This complex formation was also specifically blocked by NF-κB consensus oligonucleotide (Fig. 5C, lane 9 in the right panel). It is known that treatment with a specific antibody leads to either formation of an antibody-protein-DNA ternary complex (a supershift band) or just disruption of the protein-complex formation, resulting in a reduction of the complex band in the EMSA autoradiograph (17). Our observation of disruption of the NF-κB/DNA complex formation by anti-NF-κB antibodies without a clear supershift band is also in agreement with those reported by others (31, 32). Together, the EMSA results demonstrate that NGF enhances the complex formation between the putative NF-κB cis-element and the NF-κB p50/p65 heterodimer, suggesting that NGF activation induces translocation of the NF-κB p50/p65 heterodimer to the nucleus to bind to the putative NF-κB cis-element on the dor promoter. The EMSA results are also consistent with our earlier results from the reporter gene assays, in which we found that NGF activated NF-κB signaling, and both the NF-κB super inhibitor IκBαMo and the nuclear translocation specific inhibitor SN50 blocked NGF activation of dor expression (Fig. 4).

Knockdown of NF-κB p65 Protein by NF-κB p65 siRNA Resulted in a Reduction in dor Expression—To confirm that the NF-κB pathway is required for NGF/Trk/PI3K/Akt and NF-κB signaling-dependent dor expression, using the RNA interference method we examined the effect of the NF-κB p65 expression level on NGF-induced dor expression. Introduction of short interference NF-κB p65 RNA duplex pool into PC12h cells resulted in about a 50% reduction in the p65 protein level (p = 0.0004), whereas the mock transfection agent and scrambled non-specific RNA control pool did not have a significant effect on the level of NF-κB p65 protein (Fig. 6A, left and middle panels). The same treatment of PC12h cells with p65 siRNA resulted in a reduction in dor expression in the left panel, an RT-PCR analysis of the dor mRNA level in cells treated with mock or control siRNA pool and p65-specific siRNA pool for 48 h. The middle panel shows the semi-quantified results from three experiments (**, p < 0.01). In the right panel, the results from the four individual p65-specific siRNA duplexes obtained under the same conditions as the p65-specific siRNA pool was used.

DISCUSSION

The data presented here indicate that one molecular mechanism by which NGF induces dor expression is mainly via sustained activation of PI3K/Akt signaling, which leads to the activation of the transcriptional factor NF-κB to induce dor expression. Our results not only provide a novel mechanistic explanation of intracellular signaling for dor expression in PC12h cells but also imply a role of the critical NGF/TrkA/PI3K/
Akt survival signaling axis in the temporal and spatial expression of dor during neuronal differentiation.

Several NGF/TrkA downstream signaling pathways control NGF-induced molecular events in the cell (7), including PI3K, phospholipase Cγ, protein kinase C, and MAPK (7). The results from inhibitor-reporter activity profiling studies indicate that PI3K is a main player involving NGF-induced dor expression (Fig. 2). NGF treatment resulted in sustained activation of Akt (Fig. 3), which subsequently led to the activation of NF-κB by phosphorylation of IkB (Fig. 3). PI3K/Akt signaling plays a critical role in the survival of neuronal cells (7). NGF treatment of PC12 cells results in the up-regulation of the DOR protein and messenger RNA (5, 6). Ample evidence in the literature has demonstrated that the DOR agonists are involved in neuronal cell survival and neuronal differentiation. Exposure to opiates affects brain development, cell growth, and in vitro cell differentiation (34). The DOR protein and its peptide agonists promote the survival of neurons in the central nervous system and the preserved peripheral organs via an unknown mechanism (11). Moreover, treatment of PC12 cells with opioids can affect neuronal differentiation (35) and cell survival (36). The 6-opioid receptor expression pattern is temporal and spatial during neuronal development (1, 2). The opioid ligands can activate PI3K/Akt survival signaling in the opioid receptor-transfected HEK293 cells (12). Moreover, NGF is required for differentiation and maintenance of peripheral sympathetic neurons and certain nociceptive neurons (7). Furthermore, NGF, TrkA, NF-κB, and dor are all expressed in some neuronal cell types such as dorsal root ganglion neurons during development (1, 37, 38).

In certain neuronal cell types such as dorsal root ganglion neurons, the expression of NGF and its receptor decreases or disappears during the late phase of the pre- or postnatal stage of development (38). Thus, our experimental finding that the dor gene expression is directly controlled by NGF/PI3K/Akt/NF-κB survival signaling implies that temporal and spatial expression of the dor gene may play a survival role through activation of PI3K/Akt and NF-κB signaling in those DOR-expressing neurons during and after neuronal differentiation.

NF-κB is a critical transcription factor in the development and maintenance of the immune system (39). The importance of NF-κB in the central nervous system and peripheral nervous system has also been recognized recently (40, 41). For instance, activation of NF-κB in Schwann cells is required for peripheral myelin formation (42). Mice lacking NF-κB/p65 show a selective learning deficit in the spatial maze test (43). Although activation of NF-κB by certain cytokines such as tumor necrosis factor α has been well established (44), the molecular mechanism for NGF activation of NF-κB signaling in parental PC12 cells are not consistent from a variety of reports. For instance, recently Rojo et al. (45) showed that NGF activates NF-κB signaling by phosphorylation of IκBα in parental PC12 cells, which is consistent with the result from the inhibition of proteasome activity (46); however, Bui et al. (47) showed that NGF has no effect on phosphorylation of IκBα at serines 32 and 36 but does on phosphorylation of IκBα at tyrosine 42. In this study, using a defined serum-free medium to avoid the unknown effects from the serum medium previously accounted (6), we demonstrated that NGF-induced dor expression is NF-κB signaling-dependent. NGF treatment resulted in activation of Akt, leading to phosphorylation of IκBα (Fig. 3), which is in agreement with the results of Rojo et al. (45) and Maggarwar et al. (46). Interestingly, although NGF-induced phosphorylation of IκBα returned to the basal level after 4 h of NGF treatment, the level of IκBα phosphorylation was further reduced by about 40% after LY treatment for 48 h (Fig. 3C). Moreover, NGF activation of NF-κB activity was significantly reduced by expression of the dominant-negative IκBα mutant IκBαM (Fig. 4B). A parallel result was observed by analyzing the activity of dor promoter (Fig. 4B). Treatment with the nuclear translocation NF-κB-specific inhibitor, SN50, blocked NGF-mediated up-regulation of dor mRNA (Fig. 4C). These results are consistent with those from the EMSA analyses from which our data showed that NGF induced the DNA/NF-κB p50/p65 complex formation (Fig. 5). Overexpression of NF-κB p65 enhanced dor expression, and the enhancing effect was abolished by coexpression of the NF-κB super inhibitor IκBαM (Fig. 4A). RNA interference results further confirmed that the transcription factor p65 is required for dor expression in NGF-treated PC12h cells (Fig. 6). Thus, our data demonstrate that PI3K/Akt- and IκBα-regulated NF-κB signaling mediates dor expression in PC12h cells.

Regulation of the NF-κB transactivation function is mediated at several levels, including its interactions with cofactors (48). Those yet to be identified cofactors may act in concert either synergistically or antagonistically with NF-κB to regulate dor transcription. To coordinate all components of the transcriptional machinery in the nucleus to function in the right time and right place would require a concerted timing for opening the chromatin structure and modifying all necessary components, including phosphorylation and acetylation of certain transcription activators or repressors (49). A slow dephosphorylation of p-Akt as observed in Fig. 3 provides the long duration of activating signal required for completing such modifications involved in the transcriptional machinery. The duration of NF-κB activation required for switching on an NF-κB-dependent gene is varied from a few minutes (50) to more than 24 h (51). Our results show that activation of Akt and NF-κB occurred about 15 min (Fig. 3A) and that 48 h later there was still a significant amount of both p-Akt and p-IκBα in the NGF-treated neuronal cell (Fig. 3). The PI3K inhibitor LY294002 blocked phosphorylation of Akt, resulting in a reduction in p-IκBα for 48 h and the blockage of NGF-induced dor expression. Such long duration of Akt and NF-κB activation explains why it needs at least a 4-h NGF treatment to detect the expression of dor (Fig. 1C). The long duration of activation of Akt/NF-κB signaling may indicate a general mechanism for NF-κB induced late phase gene expression because analysis of transcription using activating protein 1-, serum response element-, and cyclic AMP response element-Luc reporter constructs indicates that NGF may regulate these promoters through persistent TrkA activity in differentiated PC12 cells (52). Taken together, the data from this study demonstrate an intracellular signaling mechanism of NGF-induced dor expression in NGF-treated PC12h cells as follows: binding of NGF to TrkA initiates a sustained activation of PI3K/Akt signaling, which subsequently activates the IκBα-mediated NF-κB signaling complex; translocation of the NF-κB p50/p65 complex to the nucleus to bind to the dor promoter provides the switching-on signal, which together with other yet to be identified transcriptional cofactors turns on dor expression.

To understand the function of a neuronal gene of interest, it is important to understand how the gene is switched on during neuronal differentiation. NGF induces many genes in the PC12 model system (4). This model is highly parallel with neuronal development in the brain (3, 53) where NGF also controls the temporal and spatial expression of many neuronal genes during development (7). Most studies using this system focus on the immediate early phase genes. In addition to the dor gene, NGF also mediates other G protein-coupled receptor gene products in PC12 cells, including pituitary adenylyl cyclase-activating polypeptide receptor 1 (54), M4 muscarinic acetylcholine receptor (55), and adenosine A2A receptor (56). Ras/MAPK signaling is involved in the regulation of pituitary adenylyl cyclase-activating polypeptide receptor 1 promoter activity (54) and M4 muscarinic receptor mRNA stability (55) and recently, NGF down-regulation of adenosine A2A receptor mRNA has been reported through both p75/NF-κB and TrkA/Ras/MAPK signaling pathways (56, 57). Thus, it appears that both Ras/MAPK and PI3K/Akt signaling pathways are involved in the regulation of GPCRs.
and the late phase gene products. Each signaling pathway controls the regulation of different gene products. Thus, our findings from the studies of the dor gene, which is a late phase gene, will be instructive for understanding the molecular mechanisms of NGF regulation of both other late phase neuronal genes and GPCRs.

Finally, the cellular control of the dor gene products plays an important role in opioid functions in vivo based on the dor gene-null animal and pharmacological studies (58–60). The opioid receptor activities are dependent upon the levels of the opioid receptors that are regulated both at the transcriptional and translational levels (61). Thus, the finding that PI3K/Akt signaling and transcription factor NF-κB signaling are involved in the dor gene expression indicates that this signaling pathway and the transcription factor NF-κB may play important roles in the opiate functions in vivo.

In summary, our findings in this study have implications both in neuronal development and opiate tolerance development. The present study has demonstrated that sustained activation of NGF/TrkA/PI3K/Akt/NF-κB signaling is involved in dor expression in PC12h cells, providing a novel mechanistic explanation of how NGF induces dor expression in PC12h cells in the context of intracellular signal transduction between the cell surface and the nucleus. To our knowledge, this may be the first example to show that sustained PI3K/Akt/IkBα-regulated NF-κB survival signaling is required for the control of NGF-induced G protein-coupled receptor gene expression during neuronal differentiation. Because neurotrophins have been shown to be involved in opiate-induced tolerance and addiction (62, 63) and because NF-κB has been implicated in regulating memory and neuroplasticity (64), it would be of interest to test whether NGF/TrkA/PI3K/Akt and NF-κB signaling plays any significant role in opiate-induced tolerance and addiction in the future.

Acknowledgments—We are grateful to Dr. Hiroshi Hatanaka for providing the PC12h cell line and to Dr. Warner C. Greene for providing the constructs pCMV4 and pCMV/p65 for this study.

REFERENCES

1. Zhu, Y., Hsu, M. S., and Pintar, J. E. (1998) J. Neurosci. 18, 2538–2549
2. Georges, F., Normand, E., Bloch, B., and Le Moine, C. (1998) Brain Res. Dev. Brain Res. 109, 187–199
3. Greene, L. A., and Tischler, A. S. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2424–2428
4. Lee, N. H., Weinstock, K. G., Kirkness, E. F., Earle-Hughes, J. A., Fuldner, R. A., Cotte, C. M., and Venter, J. C. (1995) Nat. Genet. 9, 3392–3398
5. Kunsch, C., Ruben, S. M., and Rosen, C. A. (1992) Mol. Cell. Biol. 12, 4412–4421
6. Abood, M. E., and Tao, Q. (1995) J. Biol. Chem. 270, 13585–13588
7. Sofroniew, M. V., Howe, C. L., and Mobley, W. C. (2001) Annu. Rev. Neurosci. 24, 1217–1281
8. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) J. Biol. Chem. 270, 5241–5248
9. Liu, H. C., Shen, J. T., Augustin, L. B., Ko, J. L., and Loh, H. H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10356–10365
10. Yuan, J., and Yankner, B. A. (2000) Nat. Rev. Mol. Cell Biol. 1, 752–754
11. Borlongan, C. V., Wang, Y., and Su, T. P. (1995) J. Neurosci. 15, 8199–1207
12. Ip, N. Y., Stitt, T. N., Tapley, P., Klein, R., Glass, D. J., Fandl, J., Greene, L. A., Barabid, M., and Yancopoulos, G. D. (1993) Neuron 10, 137–149
13. Tsoufas, P., Soppet, D., Escandon, E., Tessarollo, L., Mendoza-Ramirez, J. L., Rosenhall, A., Nikolaos, K., and Parada, L. F. (1993) Neuron 10, 975–990
14. Hatanaka, H. (1982) J. Biol. Chem. 257, 9238–9241
15. Chao, M. V., and Hempstead, B. L. (1995) Trends Neurosci. 18, 321–326
16. Wittert, G., Hope, P., and Pyle, D. (1996) Biochem. Biophys. Res. Commun. 228, 877–881
17. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1993) Current Protocols in Molecular Biology (Chanda, Y. B., ed) Vol. 2, John Wiley & Sons, Inc., New York