Phosphatidylinositol 3-Kinase and Akt Protein Kinase Are Necessary and Sufficient for the Survival of Nerve Growth Factor-Dependent Sympathetic Neurons

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Recent studies have suggested a role for phosphatidylinositol (PI) 3-kinase in cell survival, including the survival of neurons. We used rat sympathetic neurons maintained in vitro to characterize the potential survival signals mediated by PI 3-kinase and to test whether the Akt protein kinase, a putative effector of PI 3-kinase, functions during nerve growth factor (NGF)-mediated survival. Two PI 3-kinase inhibitors, LY294002 and wortmannin, block NGF-mediated survival of sympathetic neurons. Cell death caused by LY294002 resembles death caused by NGF deprivation in that it is blocked by a caspase inhibitor or a cAMP analog and that it is accompanied by the induction of c-jun, c-fos, and cyclin D1 mRNAs. Treatment of neurons with NGF activates endogenous Akt protein kinase, and LY294002 or wortmannin blocks this activation. Expression of constitutively active Akt or PI 3-kinase in neurons efficiently prevents death after NGF withdrawal. Conversely, expression of dominant negative forms of PI 3-kinase or Akt induces apoptosis in the presence of NGF. These results demonstrate that PI 3-kinase and Akt are both necessary and sufficient for the survival of NGF-dependent sympathetic neurons.

Key words: apoptosis; phosphatidylinositol 3-kinase; NGF; neuronal survival; Akt; neurotrophic factor

The survival of developing neurons requires extracellular signals that actively prevent programmed cell death. These signals are provided, in part, by neurotrophic factors such as nerve growth factor (NGF) (Oppenheim, 1991). Sympathetic neurons from the rat superior cervical ganglion (SCG) provide a well characterized model for studying NGF-mediated neuronal survival. If NGF is withdrawn from cultured sympathetic neurons, they undergo apoptosis (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994). Similarly, the SCG from mice lacking NGF (Crewley et al., 1994) or its receptor, TrkA (Smeyne et al., 1994), fail to develop because of massive neuronal death. In contrast, adding exogenous NGF in vivo prevents the naturally occurring death of sympathetic neurons during development (Hendry and Campbell, 1976). Thus, cultures of dissociated sympathetic neurons provide a useful in vitro model for studying neurotrophic factor dependence.

Although the function of NGF as a survival-promoting factor is well established, the mechanisms responsible for NGF-mediated survival remain, in large part, uncharacterized. NGF affects neuronal survival and differentiation by binding to and activating the TrkA tyrosine kinase receptor (Barbacid, 1994). Once activated, TrkA autophosphorylates specific tyrosine residues within its intracellular domain (Kaplan et al., 1991; Klein et al., 1991). The phosphorylated tyrosines serve as protein interaction sites for several signaling molecules, including SHC, phospholipase C-γ, and phosphatidylinositol (PI) 3-kinase (Ohmichi et al., 1991; Carter and Downes, 1992; Raffioni and Bradshaw, 1992; Soltoff et al., 1992; Obermeier et al., 1993). The consequences of TrkA activation include Shc/Grb2/Sos-dependent activation of Ras and the subsequent activation of mitogen-activated protein (MAP) kinases, phospholipase C-γ-mediated production of diacylglycerol and inositol trisphosphate, and PI 3-kinase-mediated production of 3′-phosphorylated phosphoinositides (Kaplan and Stephens, 1994).

Although previous studies have focused on Ras and MAP kinases in NGF-mediated survival (Borasio et al., 1989, 1993; Ferrari and Greene, 1994; Nobes and Tolkovsky, 1995; Xia et al., 1995; Yao and Cooper, 1995; Creedon et al., 1996; Virdee and Tolkovsky, 1996), several recent reports suggest that PI 3-kinase functions in the survival pathways initiated by certain growth factors and survival-promoting agents. Using the PI 3-kinase inhibitors wortmannin and LY294002, Yao and Cooper (1995) reported that the inhibition of PI 3-kinase activity induces apoptosis in PC12 cells in the presence of NGF. This observation since has been extended to other immortalized cell lines, particularly those dependent on insulin-like growth factor-1 (IGF-1) for survival. For example, in Rat-1 fibroblasts PI 3-kinase inhibitors block IGF-1-mediated protection from apoptosis induced by UV irradiation (Kulik et al., 1997) and serum- or IGF-1-mediated protection from c-Myc-induced apoptosis (Kaufmann-Zeh et al., 1997; Kennedy et al., 1997). Similarly, PI 3-kinase inhibitors block IGF-1-mediated survival of PC12 cells (Parrizas et al., 1997). Consistent with these results, a function for PI 3-kinase has been demonstrated recently in the survival of cerebellar granule neurons mediated by IGF-1 or by potassium depolarization (D’Mello et al., 1997; Dudek et al., 1997; Miller et al., 1997).

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To characterize further the role of PI 3-kinase in the survival of primary neurons, we have used NGF-dependent sympathetic neurons to compare the cell death caused by inhibitors of PI 3-kinase with that caused by NGF withdrawal. We also have tested whether a putative effector of PI 3-kinase, the serine/threonine protein kinase Akt (also known as protein kinase B or Rac protein kinase) (Burgering and Coffey, 1995; Franke et al., 1995; Kohn et al., 1995), functions in the survival of NGF-dependent neurons.

**MATERIALS AND METHODS**

Cycloheximide, actinomycin D, Wortmannin, and chlorophenylthio-

CAMP (cAMP) were purchased from Sigma (St. Louis, MO); boc-

aspartyl(Ome)-fluoromethylketone (BAF) was obtained from Enzyme

Systems Products (Dublin, CA); LY294002 was obtained from Biomol

Research Laboratories (Plymouth Meeting, PA). Flavopiridol was pro-

vided by D. J. Johnson and E. Sauvage (Drug Synthesis and Chem-

istry Branch, National Cancer Institute, Bethesda, MD).

**Cell culture.** Primary cultures of sympathetic neurons were prepared

from SCG of embryonic day 21 rats as previously described (Martin et al.,

1995) except that a preplating step was included to minimize non-

neuronal cells. For preplating, SCG neurons were dissociated and resus-

pended in NGF-containing media (AM50 medium) consisting of 90% Min-

imum Essential Media (MEM; Life Technologies, Gaithersburg,

MD), 10% FBS (Sigma), 2 mM glutamine, 20 μM uridine, and 20 μM

fluodeoxyuridine (to inhibit the proliferation of non-neuronal cells),

100 μM penicillin, 100 μg/ml streptomycin, and 20 ng/ml NGF (Harlan

Bioproducts, Madison, WI). The cell suspension was filtered through a

Nitex filter (size 3–20/14; Tetko, Briarcliff Manor, NY) and plated onto

Primaria tissue culture dishes (Becton Dickinson, Lincoln Park, NJ) for a

period of 1–2 hr. Nonadherent cells were collected and concentrated by

centrifugation (10 min at 450 x g) and plated onto 0.3% Triton X-100/PBS for 10 min, rinsed in PBS, fixed in fresh 4% paraformaldehyde in PBS for 15 min, destained in H2O, dehydrated in increasing ethanol concentrations, and transferred to xylene (Fisher Scientific, Pittsburgh, PA), and finally
coverlapped by using Pro-texx mounting media (Baxter Diagnostics,

Deerfield, IL). Neurons staining darker than debris with a clearly defined

cellular outline and a well defined nucleus were scored as viable. For each

experimental treatment four fields of cells from each of three to four

wells were counted under a 20x objective, and the average number of

viable cells per field was determined. This was normalized to the average

number of viable neurons in parallel nontreated control cultures. The

results reported for each condition represent the means and errors

(where appropriate) obtained from two to four independent

measurements.

**RT-PCR analysis.** Preparation of cDNAs and analysis of gene expres-

sion in SCG neurons treated with LY294002 were essentially the same as

those described for NGF-deprived neurons (Freeman et al., 1994). Pre-

plated cultures (25,000 neurons plated per time point) were maintained

in AM50 for 5 d and then treated with LY2940002 diluted to a final

concentration of 100 μM in AM50 for the indicated intervals. Poly-

denylated RNA was isolated by direct hybridization to oligo-dT-cellulose

beads, as described by the manufacturer (QuickPrep Micro mRNA

Purification Kit, Pharmacia Biotech, Piscataway, NJ). One-half of the

recovered mRNA was reverse-transcribed by using Moloney murine

leukemia virus reverse transcriptase (Superscript II RT; Life Technolo-

gies, Gaithersburg, MD) in the presence of random hexamers (16–18 μM)

as primers in reactions containing 50 mM Tris, pH 8.3, 40 mM KCl, 10 mM

DTT, 6 mM MgCl2, 20 mM RNasin (Promega, Madison, WI), and 500 μM each dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim). After a 1 hr incubation

at 42°C, the reaction was terminated by adding 80 μl of H2O and heating the reaction for 5 min at 95°C. Specific cDNAs were amplified in 30 μl PCR

reactions containing the appropriate primer pairs (0.6 μM each), 1× Taq

polymerase buffer, 1 U Taq DNA polymerase, 0.6 mM each dATP, dCTP, dGTP, and dTTP, 6 μM primer, 100 μl each dATP, dGTP, and dTTP, 6 μM primer, and 500 μM each dATP, dCTP, dGTP, and dTTP (DuPont NEN, Boston, MA), and 0.6 μl cDNA synthesized in the RT reaction.

PCR parameters were 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C for

16–28 cycles, followed by a final 10 min incubation at 72°C. Reaction

products were separated by electrophoresis and analyzed by autoradiog-

raphy (Hyperfilm phosphorImager; Amersham, Arlington Heights, IL). For

control experiments to determine the linear range of PCR amplifi-

cation and to verify the identity of amplified products were as described

previously, as were the sequences of oligonucleotide primers (Estus et al.,

1994; Freeman et al., 1994).

**Plasmid expression vectors.** Expression vectors for the Escherichia coli

β-galactosidase (lacZ) gene, p1103, and p1104kin under the control of

the human cytomegalovirus immediate early gene promoter have been

described previously (Greenlund et al., 1995a; Hu et al., 1995). Myr-Akt

and A2myr-Akt cDNAs (Kohn et al., 1996b) were cloned behind the

cyto-mammalian virus promoter in the plasmid pcDNA3 (Invitrogen, San

Diego, CA) by inserting the Km resistance fragment from pECE-my-

Akt or pECE-A2myr-Akt between the Km and Xho I sites of pcDNA3.

The Δp85 cDNA was removed from pGEX-Δp85 (Kotani et al., 1994) as

a 2189–2201 EcoRI fragment inserted into pEI plasmids (Amersham,

Buckingham, England) and Xho I sites of pcDNA3. The rat AH·Akt-Flag

construct, encoding amino acids 1–148 of rat Akt followed by the Flag

epitope, was generated by pfu polymerase (Strategen, La Jolla, CA) amplification from rat SCG cDNA, using a 5′ primer (5′-GCG GAT CCA CAA TGA ACC ACG TAG CAA TTG TG-3′) containing a BamHI site, a consensus transcrip-

tion start site, and nucleotides 1–21 of the rat Akt open reading frame

(Konishi et al., 1994). The 3′ primer (5′-GCG AAT TCT CAC TCG TGG

TCA TCG TCG TCC TTG TAG TTC ATG GTA AGC ACA GGG

TG-3′) contained an EcoRI site, nucleotides 427–444 of the rat

Akt open reading frame, and sequences corresponding to the Flag epitope.

The PCR-generated fragment was ligated into the BamHI and EcoRI sites of pcDNA3. DNA sequencing revealed that the construct was correct. AktK179A–pcDNA3 was constructed by ligating an open

EcoRI fragment of AktK179A (Kotani et al., 1994) to pcDNA3 (Invitrogen,

Buckingham, England) and Xho I sites of pcDNA3. The rat AH·Akt-Flag

construct, encoding amino acids 1–148 of rat Akt followed by the Flag

epitope, was generated by pfu polymerase (Strategen, La Jolla, CA) amplification from rat SCG cDNA, using a 5′ primer (5′-GCG GAT CCA CAA TGA ACC ACG TAG CAA TTG TG-3′) containing a BamHI site, a consensus transcrip-

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Akt open reading frame, and sequences corresponding to the Flag epitope.

The PCR-generated fragment was ligated into the BamHI and EcoRI sites of pcDNA3. DNA sequencing revealed that the construct was correct. AktK179A–pcDNA3 was constructed by ligating an EcoRI/ BamHI·Flag fragment from pSG5 HA·PKB K179A (Burgering and Coffey, 1995), containing an Akt kinase-inactive mutant cDNA, into the EcoRI and EcoRV sites of pcDNA3.

**Intracellular microinjections.** Neurons plated on poly-l-ornithine-

coated glass-bottomed 35 mm dishes were microinjected by using a Nikon Diaphot 300 inverted microscope.

**Quantification of neuronal viability.** Equal numbers of neurons plated on

collagen-coated two-well chamber slides were subjected to the appropri-

ate treatments and then were fixed with fresh 4% paraformaldehyde/PBS

overnight at 4°C. Neurons were rinsed in PBS and stained briefly with

0.1% crystal violet (EM Science, Gibbstown, NJ). The neurons were

dehydrated in H2O, dehydrated and stained to visualize the nuclei

of cells. After two additional rinses with PBS, the slides were covered

with glass coverslips, using a mounting solution of 50% glycerol and 0.1%

phenylene-diamine in PBS, and then visualized by fluorescence microsco-

py with a Nikon Diaphot 300 inverted microscope.

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potassium phosphate, pH 7.4) containing 4 mg/ml rhodamine-dextran (10 Kz; Sigma) to mark the injected cells. For microinjections, neurons were maintained in AM50 for 5–6 d and then transferred to Leibovitz's L-15 medium (Life Technologies) immediately before injection. Approximately 48 hr later the cells were stained with the DNA-binding dye Hoechst 33342 in L-15 medium and evaluated for survival. Neuronal viability was assessed by counting the number of rhodamine-positive cells that were phase-bright with smooth and intact neurites, a discernible nucleus, and diffuse and homogeneous chromatin. A small percentage of injected neurons did not regain membrane integrity and died within a few hours of injection; these neurons did not affect subsequent analyses. The percentage of survival was equal to the number of viable cells remaining after NGF deprivation (determined as described above) divided by the number of rhodamine-positive cells counted before NGF withdrawal. For each plasmid the results reported were derived from at least three independent experiments involving a minimum of 200 injected neurons per plasmid per experiment. In all microinjection experiments a blinded observer accessed cell viability. Immunofluorescence for expression of p110α (Crowder and Freeman, 2000), A2myr-Akt, AKT-Flag, and AktK179A in injected neurons was confirmed by using indirect immunofluorescence. In each case the neurons were microinjected with solutions containing 50 mg/ml expression vector DNA and 2 mg/ml lysine-fixable tetramethylrhodamine-dextran dye (Molecular Probes) in KPi buffer. Injected neurons were incubated with DNA and 2 mg/ml lysine-fixable tetramethylrhodamine-dextran dye (Molecular Probes) in KPi buffer. Then the cells were incubated with the selective PI 3-kinase inhibitor LY294002 (Fig. 1). LY294002-treated neurons have shrunken cell soma and fragmented neurites and frequently contain one or more compact spheres of condensed chromatin in their nuclei, in contrast to the uniformly dispersed chromatin present in the nuclei of nontreated neurons (Fig. 1B,E). Many of the nuclei are labeled by the TUNEL assay, indicating the presence of DNA strand breaks (Fig. 1F). These characteristics of LY294002-treated neurons are indistinguishable from those that typify apoptosis caused by NGF deprivation (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994).

Despite these morphological similarities, SCG neurons treated with LY294002 die more slowly than neurons deprived of NGF (Fig. 2A). In control cultures the removal of NGF caused ~60% death by 24 hr and >75% death by 48 hr. In contrast, 34% of neurons treated with 100 μM LY294002 in the presence of NGF died by 48 hr, with 85% dying by 96 hr. Moreover, death of LY294002-treated neurons commences only after at least 24 hr, as compared with a lag period of 15–18 hr for NGF deprivation-induced death (Deckwerth and Johnson, 1993). In contrast to these results, death of cerebellar granule neurons caused by LY294002 treatment occurs at the same rate as death caused by the withdrawal of survival factors (Miller et al., 1997). Thus, the increased rate of death caused by NGF withdrawal relative to LY294002 treatment may indicate that factors other than the inactivation of PI 3-kinase are rate-determining for the death of sympathetic neurons after NGF removal.

Under our experimental conditions LY294002 inhibited NGF-mediated survival at concentrations as low as 10 μM, with a 50% inhibitory concentration (IC50) of ~30 μM (Fig. 2B). Although the IC50 of LY294002 for blocking PI 3-kinase activity in vitro is 1.4 μM (Vlahos et al., 1994), concentrations ranging from 10 to 100 μM often are necessary to inhibit PI 3-kinase in intact cells (Vlahos et al., 1994; Yao and Cooper, 1996; Miller et al., 1997). Wortmannin, another PI 3-kinase inhibitor (Yano et al., 1993), also blocked the survival-promoting effects of NGF on sympathetic neurons (data not shown); the time course was similar to that of LY294002 and death was virtually complete at a concentration (100 μM) previously shown to be necessary for efficiently blocking PI 3-kinase activity and inducing DNA fragmentation in NGF-treated PC12 cells (Yao and Cooper, 1995). The ability of two structurally distinct inhibitors of PI 3-kinase to block NGF-mediated survival strongly implicates PI 3-kinase, or a PI 3-kinase-related enzyme, as a necessary transducer of the survival signals initiated by NGF in primary neurons.

A variety of pharmacological agents can inhibit the death of
NGF-deprived sympathetic neurons. These include the protein synthesis inhibitor cycloheximide and the RNA synthesis inhibitor actinomycin D (Martin et al., 1988), cell-permeable cAMP analogs (Rydel and Greene, 1988), the cyclin-dependent kinase inhibitor flavopiridol (Park et al., 1996), membrane-depolarizing concentrations of extracellular potassium (Koike et al., 1989), and the nonselective caspase inhibitor BAF (Deshmukh et al., 1996).

We tested several of these agents for their ability to inhibit LY294002-induced death (Fig. 3). The addition of either BAF (100 μM) or cpt-cAMP (300 μM) in large part prevented the death of LY294002-treated neurons. The addition of actinomycin D also provided protection from cell death, albeit to a lesser extent. Although the cell bodies of neurons rescued by BAF, cpt-cAMP, or actinomycin D remained phase-bright with clearly discernible nuclei and nucleoli, significant neuritic degeneration continued to occur in these cultures (data not shown), suggesting that the mechanisms that maintain neurite integrity may be distinct from those that control cell survival. In contrast to the above reagents, flavopiridol (1 μM) provided little protection against LY294002-induced death, whereas LY294002 treatment of potassium-depolarized neurons resulted in even greater cell death than exposure to LY294002 in the presence of NGF. The ability of cpt-cAMP, BAF, and actinomycin D to prevent death caused either by NGF withdrawal or LY294002 suggests that both treatments activate a similar cell death pathway.

Withdrawal of NGF from sympathetic neurons results in increased mRNA expression of a select subset of genes, including c-jun, c-fos, and cyclin D1 (Estus et al., 1994; Freeman et al., 1994). RT-PCR analysis of mRNAs isolated from LY294002-treated and nontreated cultures demonstrated that the expression of c-fos, c-jun, and cyclin D1 increases during LY294002-induced death (Fig. 4). Whereas c-jun expression exhibited a relatively constant prolonged elevation, c-fos expression increased sharply between 25 and 30 hr. cyclin D1 message levels exhibited a sustained elevation (three- to fourfold), peaking after 30 hr of treatment. As expected, LY294002 treatment led to a reduction in the abundance of the ubiquitously expressed cyclophilin mRNA and in the neuronally expressed tyrosine hydroxylase and p75 neurotrophin receptor mRNAs. Unlike NGF deprivation (Freeman et al., 1994), LY294002 treatment resulted in a steady decrease in the mRNA level of the Schwann cell marker S100β, suggesting that LY294002 also may be detrimental to certain non-neuronal cells present at low levels in these cultures. The induction of c-fos, c-jun, and cyclin D1 during neuronal death induced by inhibiting PI 3-kinase or by the withdrawal of NGF provides further evidence that LY294002-induced death and NGF deprivation-induced death share a common mechanism.

**NGF-stimulated Akt protein kinase activity is blocked by PI 3-kinase inhibitors**

The Akt protein kinase is activated by a variety of growth factors via a PI 3-kinase-dependent pathway (Burgering and Coffer,
Akt has been implicated in transducing growth factor and extracellular matrix-dependent survival signals in fibroblast, epithelial, and lymphoid cell lines (Ahmed et al., 1997; Kauffmann-Zeh et al., 1997; Kennedy et al., 1997; Khwaja et al., 1997; Kulik et al., 1997). Recently, a role for Akt also has been defined in the survival of rat cerebellar granule neurons (Dudek et al., 1997). To assess a potential function for Akt in NGF-mediated survival of sympathetic neurons, we first tested whether endogenous Akt protein kinase activity is stimulated in neurons treated with NGF, using histone H2B as an in vitro substrate (Fig. 5). After 15 min of NGF stimulation, Akt protein kinase activity increased approximately threefold above the basal level observed in the absence of NGF. Treatment with 100 μM LY294002 (or 100 nM wortmannin; data not shown) reduced the NGF-stimulated kinase activity to the level observed in NGF-deprived neurons. In contrast, treatment of neurons with 10 μM LY294002 caused only a partial (50–60%) reduction in NGF-stimulated Akt kinase activity (data not shown). Thus, NGF treatment leads to the activation of endogenous Akt protein kinase activity in sympathetic neurons, which can be blocked by inhibitors of PI 3-kinase at concentrations similar to those that block survival.

Expression of activated PI 3-kinase or activated Akt prevents the death of NGF-deprived neurons

The results described above indicate that NGF stimulates a PI 3-kinase-regulated pathway that leads to Akt activation. To test whether the activation of this pathway in the absence of NGF would be sufficient to promote neuronal survival, we microinjected sympathetic neurons with plasmid DNAs expressing either a constitutively active form of PI 3-kinase (p110*) or a kinase-inactive mutant (p110*Δkin) (Hu et al., 1995; Kulik et al., 1997) (Fig. 6). Indirect immunofluorescence analysis of microinjected neurons verified that p110* and p110*Δkin were overexpressed successfully in 85–90% of injected cells. After 48 hr of NGF deprivation, most neurons injected with p110* maintained a phase-bright cell soma with uniformly dispersed chromatin and intact neurites (Fig. 7A–C). In contrast, the majority of neurons injected with β-galactosidase (LacZ) or p110*Δkin and then...
only histone H2B, entries. Relative changes in the mRNA levels of specific genes were measured by semiquantitative RT-PCR analysis, as outlined in Materials and Methods. Shown are representative results from one of three independent time courses, all of which yielded similar results. PCR cycle numbers for each of the genes were as follows: cyclophilin, 18 cycles; tyrosine hydroxylase (TOH), 18 cycles; p75 neurotrophin receptor (p75), 18 cycles; c-fos, 24 cycles; c-jun, 24 cycles; cyclin D1, 25 cycles; S100β, 28 cycles.

Figure 4. Expression of cyclin D1, c-jun, and c-fos increase during LY294002-induced death. Neurons cultured in AM50 for 5 d received no treatment (0 hr) or were treated with 100 μM LY294002 in AM50 for the indicated time intervals. Relative changes in the mRNA levels of specific genes were measured by semiquantitative RT-PCR analysis, as outlined in Materials and Methods. Shown are representative results from one of three independent time courses, all of which yielded similar results. PCR cycle numbers for each of the genes were as follows: cyclophilin, 18 cycles; tyrosine hydroxylase (TOH), 18 cycles; p75 neurotrophin receptor (p75), 18 cycles; c-fos, 24 cycles; c-jun, 24 cycles; cyclin D1, 25 cycles; S100β, 28 cycles.

Figure 5. NGF stimulates Akt protein kinase activity in a PI 3-kinase-dependent manner. Akt protein kinase activity was analyzed in immune complex kinase assays prepared from SCG neurons treated as follows: lane 1, neurons deprived of NGF for 10 hr; lane 2, neurons deprived of NGF for 10 hr and then stimulated with NGF for 15 min; lane 3, neurons deprived of NGF for 10 hr and then treated with NGF for 15 min in the presence of 100 μM LY294002; lane 4, mock kinase reaction containing only histone H2B, [γ-32P]ATP, and protein A beads. Reaction products were analyzed by SDS-PAGE, followed by autoradiography and Phosphor-Imager analysis. Akt protein kinase activity increased an average of 3.1-fold after NGF stimulation (n = 4).

Deprived of NGF were morphologically indistinguishable from uninjected NGF-deprived cells and either contained condensed chromatin or lacked detectable chromatin (Fig. 7D–F). Quantifying the cell survival (see Materials and Methods) revealed that expression of p110β resulted in the survival of 78% of injected neurons after 48 hr of NGF deprivation, as compared with 23% survival for control injections. A2myr-Akt provided an intermediate, but significant, level of protection (46% survival) against NGF withdrawal. Thus, overexpression of activated forms of either PI 3-kinase or Akt protein kinase effectively prevents the death of NGF-deprived neurons. These results raise the possibility that NGF promotes the survival of sympathetic neurons, at least in part, via a PI 3-kinase-regulated pathway that involves Akt.

Figure 6. Constitutively active PI 3-kinase (p110β) is expressed efficiently in microinjected sympathetic neurons. Neurons were injected with p110β plasmid and lysine-fixable tetramethylrhodamine-dextran. At 15 hr after microinjection the neurons were fixed and stained with the 9E10 monoclonal antibody, which recognizes the myc epitope attached to p110β. The 9E10 antibody was detected by FITC-conjugated anti-mouse antibodies. A shows rhodamine-labeled (injected) cells. B, Immunofluorescence analysis shows that all of the injected neurons in A overexpress p110β. Overall, p110β was detected in 90% of the injected neurons. Scale bar, 30 μm.

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Dominant negative forms of PI 3-kinase and Akt induce neuronal death in the presence of NGF

Although LY294002 and wortmannin induce apoptosis in NGF-maintained neurons, the specific targets of these drugs, at the concentrations used here, cannot be identified. As an independent means of addressing whether PI 3-kinase is required for NGF-mediated survival, we microinjected NGF-maintained neurons with a plasmid expressing a dominant negative form of PI 3-kinase. The PI 3-kinase enzyme activated by tyrosine kinase receptors such as TrkA consists of a p110 catalytic subunit and a p85 regulatory subunit. The dominant negative mutant (Δp85) contains a deletion within the inter-SH2 domain of p85 that abolishes its binding to p110, but not to growth factor receptors

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Thus, Δp85 competes with wild-type p85 for binding to activated receptors, but it is unable to induce p110 catalytic activity. Expression of Δp85 in neurons maintained in the presence of NGF resulted in a significant decrease in survival, as compared with control neurons microinjected with a LacZ expression vector (Fig. 10A). These data, together with the results obtained by using PI 3-kinase inhibitors, indicate that PI 3-kinase is required for the survival of sympathetic neurons by NGF.

Expression of either a truncated form of Akt consisting of residues 1–147 (AH-Akt) or a kinase-inactive Akt mutant (AktK179A) induces cell death in insulin-treated cerebellar granule cells and adherent epithelial cells (Dudek et al., 1997; Khwaja et al., 1997). In these studies the dominant negative nature of the mutants was demonstrated by a reduction in growth factor-stimulated Akt kinase activity that occurred in cells cotransfected with mutant and wild-type Akt. To test whether Akt is necessary for NGF-promoted survival, we microinjected NGF-maintained neurons with expression plasmids containing either an epitope-tagged version of AH-Akt or AktK179A (Fig. 10B). Expression of either AH-Akt or AktK179A in the presence of NGF substantially increased the number of apoptotic neurons, as compared with control neurons expressing LacZ. The extent of cell death in neurons expressing AH-Akt or AktK179A in the presence of NGF was slightly greater than that observed in neurons treated with LY294002 for 3 d (see Fig. 2). In control experiments the expression of AktK179A did not affect cell death caused by NGF deprivation. These results show that Akt, in addition to PI 3-kinase, is required for NGF-mediated neuronal survival.

**DISCUSSION**

Recent studies have implicated PI 3-kinase as an intracellular transducer of survival signals initiated by various growth factors. We began this study by asking whether PI 3-kinase also functions in the NGF-dependent survival of primary neurons. We found that neuronal death caused by inhibition of PI 3-kinase shares several features with death caused by NGF deprivation, suggesting that PI 3-kinase may function as an essential mediator of survival in NGF-dependent neurons. Consistent with this, expression of activated PI 3-kinase rescues NGF-deprived neurons from cell death, whereas expression of dominant negative PI 3-kinase blocks survival in the presence of NGF. NGF treatment of neurons activates Akt, a putative effector of PI 3-kinase, and Akt activation is dependent on PI 3-kinase. Expression of dominant negative forms of Akt block NGF-promoted survival, whereas expression of activated Akt is sufficient for survival in the absence of NGF. Taken together, these results identify Akt as an important mediator of PI 3-kinase-dependent survival signals initiated by NGF in sympathetic neurons.

The neuronal death caused by PI 3-kinase inhibitors resembles, in part, the death caused by NGF withdrawal, suggesting that both stimuli may activate similar cell death pathways. In both cases the death exhibits features characteristic of apoptosis, including cellular atrophy, chromatin condensation, TUNEL reactivity, and neurite fragmentation. The caspase inhibitor BAF and the RNA synthesis inhibitor actinomycin D block death caused by either treatment, suggesting a shared requirement for caspase activation and certain transcriptional events. Likewise, cpt-cAMP treatment blocks death caused by PI 3-kinase inhibition or NGF.
over, rapamycin (0.01–100 nM) does not block the survival of (Carter and Downes, 1992; Raffioni and Bradshaw, 1992). Moreover, only p85/p110 PI 3-kinase is known to be activated by NGF (Stephens et al., 1994; Hartley et al., 1995; Brunn et al., 1996). Of the PI 3-kinase superfamily. Among these are the tyrosine kinase-activated p85/p110 PI 3-kinases, heterotrimeric G-protein-stimulated p110α, and the mammalian target of rapamycin (mTOR) kinase (Stephens et al., 1994; Hartley et al., 1995; Brunn et al., 1996). Of these, only p85/p110 PI 3-kinase is known to be activated by NGF (Carter and Downes, 1992; Raffioni and Bradshaw, 1992). Moreover, rapamycin (0.01–100 nM) does not block the survival of NGF-maintained sympathetic neurons (R. Freeman, unpublished observation), suggesting that mTOR is not necessary for NGF-mediated survival. Finally, the expression of a dominant negative mutant of p85 blocked NGF-promoted survival. Thus, although we cannot exclude the possibility of other effects, the inhibitors used in this study most likely inhibit the activation of p85/p110 PI 3-kinase by NGF.

Expression of activated PI 3-kinase or activated Akt is sufficient to keep neurons alive in the absence of NGF. The degree of saving conferred by activated PI 3-kinase and activated Akt is similar to that obtained by overexpressing the Bcl-2 protein in sympathetic neurons (Garcia et al., 1992; Greenlund et al., 1995b) (R. Crowder, unpublished observation). The form of PI 3-kinase that we used consists of the p110 catalytic subunit of PI 3-kinase fused at its N terminus to the inter-SH2 domain of the p85 regulatory subunit. Although expression of this and similar forms of activated PI 3-kinase can suppress apoptosis under certain conditions (Kaufmann-Zeh et al., 1997; Kennedy et al., 1997; Khwaja et al., 1997; Kulik et al., 1997), the mechanism by which this occurs is poorly characterized. When the activated form of PI 3-kinase used in our experiments was transiently expressed in COS-7 cells, Akt and pp70s6 kinase were activated constitutively in the absence of growth factor stimulation (Hu et al., 1995; Klippel et al., 1996). Although it is not known whether the expression of activated PI 3-kinase in neurons leads to activation of Akt, our data indicating that Akt is sufficient for survival in the absence of NGF and necessary for NGF-dependent survival suggest that Akt activation may be a critical event.

The activated form of Akt used in our experiments lacks its phospholipid-binding PH domain but is targeted to the cell membrane via a myristoylation sequence added to its N terminus (Kohn et al., 1996b). In fibroblasts, COS-7 cells, and cerebellar granule cells the overexpression of Akt or membrane-targeted forms of Akt also inhibit cell death (Dudek et al., 1997; Kaufmann-Zeh et al., 1997; Kennedy et al., 1997; Kulik et al., 1997). In these studies full-length Akt (with its PH domain intact)
was expressed rather than the PH domain-minus form used in our experiments. Assuming that the survival that occurs after expression of myr-Akt (lacking the PH domain) is not dependent on endogenous wild-type Akt, then the binding of phospholipids or other interactions mediated by the PH domain would not appear to be necessary for myr-Akt to promote survival in neurons.

The ability of activated Akt to prevent apoptosis after NGF withdrawal, taken together with the increase in Akt kinase activity that occurs after NGF stimulation, suggests that Akt may function to transduce NGF-initiated survival signals in neurons. To demonstrate more directly a role for Akt in NGF-mediated survival, we tested the effects of functionally inhibiting Akt with dominant negative mutants of Akt. Expression of either the N-terminal AH domain of Akt or a kinase-inactive Akt mutant blocked survival promotion by NGF. Because activation of Akt by NGF is prevented by LY294002 and wortmannin, these results identify Akt as a downstream target of PI 3-kinase and TrkA in the survival-promoting pathway initiated by NGF.

Although our studies demonstrate a role for Akt and PI 3-kinase in survival, they do not rule out the possibility that NGF activates additional survival pathways. The delayed onset of cell death, the slower rate of death, and the prolonged expression of c-jun and cyclin D1 caused by inhibitors of PI 3-kinase (relative to NGF withdrawal) may indicate that other survival pathways not affected by LY294002 or wortmannin might be downregulated after the removal of NGF. Turning off such pathways by withdrawing NGF could lead to faster cell death by (1) more completely inactivating the PI 3-kinase pathway than is possible with LY294002 or wortmannin in this system or (2) inactivating additional or alternative survival pathways. Studies in PC12 cells suggest that certain MAP kinases, in particular the extracellular signal-regulated kinases (ERKs), may be important for survival mediated by NGF or other growth factors (Xia et al., 1995). However, recent studies using sympathetic neurons demonstrate that inhibitors of MAP kinase kinase-1, an upstream activator of ERKs, do not block NGF-mediated survival (Creedon et al., 1996; Virdee and Tolkovsky, 1996), suggesting that ERK activation may be dispensable for survival promotion by NGF. Previous studies have implicated a Ras-dependent pathway in the NGF-mediated survival of chick sensory neurons (Borasio et al., 1989, 1993) and rat sympathetic neurons (Nobes and Tolkovsky, 1995; Nobes et al., 1996). In both cases the introduction of Ras-neutralizing antibodies into dissociated neurons blocks survival in the presence of NGF, whereas the introduction of activated Ras results in NGF-independent survival. Known or putative effectors of Ras include the Raf/MAP kinase pathway, PI 3-kinase, Ras-GDS, and the Ras-related small GTPases, Rac and cdc42 (Marshall, 1996). Because both Ras and PI 3-kinase now have been implicated in survival promotion by NGF and because PI 3-kinase is a possible effector of Ras, these two proteins may lie within the same survival pathway.

Besides inactivating additional survival molecules, NGF withdrawal may lead to the activation of proapoptotic pathways that either are not activated or are activated incompletely after PI 3-kinase inhibition. JNK and the related kinase p38 are critical mediators of apoptosis in NGF-deprived PC12 cells (Xia et al., 1995). JNK also is activated after NGF withdrawal in sympathetic neurons, and c-Jun, a target of JNK and p38 phosphorylation, is required for neuronal death caused by NGF withdrawal (Estus et al., 1994; Ham et al., 1995; Virdee et al., 1997). Activation of JNK or p38 by NGF withdrawal, but not after inhibition of PI 3-kinase,
could contribute to the increased rate of death caused by withdrawal of NFG.

In summary, our results suggest that survival of sympathetic neurons mediated by NFG is dependent on a PI 3-kinase and Akt-regulated pathway. Other relevant participants in this survival pathway are unknown but could include Ras, phosphatidylinositol-dependent kinase-1 (Alessi et al., 1997), and two downstream targets of Akt—glycogen synthase kinase-3 (Cross et al., 1995) and Bad (Datta et al., 1997; del Peso et al., 1997).

REFERENCES


