

# Insulin-Like Growth Factor and Potassium Depolarization Maintain Neuronal Survival by Distinct Pathways: Possible Involvement of PI 3-Kinase in IGF-1 Signaling

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Cultured cerebellar granule neurons die by apoptosis when switched from a medium containing an elevated level of potassium ( $K^+$ ) to one with lower  $K^+$  (5 mM). Death resulting from the lowering of  $K^+$  can be prevented by insulin-like growth factor (IGF-1). To understand how IGF-1 inhibits apoptosis and maintains neuronal survival, we examined the role of phosphoinositide 3-kinase (PI 3-kinase). Activation of PI 3-kinase has been shown previously to be required for NGF-mediated survival in the PC12 pheochromocytoma cell line. We find that in primary neurons, IGF-1 treatment leads to a robust activation of PI 3-kinase, as judged by lipid kinase assays and Western blot analysis. Activation of PI 3-kinase is likely to occur via tyrosine phosphorylation of the insulin receptor substrate protein. Treatment with two chemically distinct inhibitors of PI 3-kinase, wortmannin and LY294002, reduces PI 3-kinase activation by IGF-1 and inhibits its survival-promoting activity, suggesting

that PI 3-kinase is necessary for IGF-1-mediated survival. Death resulting from PI 3-kinase blockade is accompanied by DNA fragmentation, a hallmark of apoptosis. Furthermore, neurons subjected to PI 3-kinase blockade can be rescued by transcriptional and translation inhibitors, suggesting that IGF-1-mediated activation of PI 3-kinase leads to a suppression of “killer gene” expression. In sharp contrast to IGF-1, elevated  $K^+$  does not activate PI 3-kinase and can maintain neuronal survival in the presence of PI 3-kinase inhibitors. Therefore, survival of granule neurons can be maintained by PI 3-kinase dependent (IGF-1-activated) and independent (elevated  $K^+$ -activated) pathways.

*Key words:* cerebellar granule neurons; phosphoinositide 3-kinase; apoptosis; insulin-like growth factor-1; elevated potassium; neuronal survival

During development of the vertebrate nervous system, approximately half of the neurons that are generated die by a process called “programmed cell death.” This naturally occurring process is mediated by apoptosis, a specific form of programmed cell death that has characteristic morphological and biochemical features (for review, see Oppenheim, 1991; Johnson and Deckworth, 1993). A key determinant of which neurons survive during this developmental period is the availability of neurotrophic factors, a class of growth factors generally secreted by targets of neuronal innervation (Oppenheim, 1991; Johnson and Deckworth, 1993). Several such factors have been identified in mammalian systems in recent years, and these include the neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3, neurotrophin-4/5, insulin-like growth factor-1 (IGF-1), and the fibroblast growth factors (FGFs).

Although neurotrophic factors are most often associated with their ability to support survival, they are also involved in a variety of other neuronal processes such as differentiation, plasticity, maintenance of specific neuronal functions, and the regulation of neuronal cell fate and precursor proliferation (for review, see Schlessinger and Ullrich, 1992). The mechanisms by which growth

factors generate such diverse actions remain unclear and the subject of intense investigation. The receptors for a number of these factors have intrinsic tyrosine kinase activity. Furthermore, recent studies have shown that the intracellular kinase regions of these receptors possess distinct motifs capable of specifically interacting with a repertoire of effector proteins containing src homology 2 (SH2) and src homology 3 (SH3) domains (for review, see Chao, 1992; Schlessinger and Ullrich, 1992; Schlessinger et al., 1992; Kapeller and Cantley, 1994). Differential interaction of the receptor tyrosine kinase with SH2 proteins therefore may provide one mechanism by which some diversity in growth factor action may be generated (Valius and Kazlauskas, 1993; Obermeier et al., 1994). Cell-specific expression of receptor tyrosine kinases and various SH2-containing proteins may also explain why a given growth factor is capable of inducing distinct or even opposite effects in different cell types (Cordon-Cardo et al., 1991). Other domains, such as PTB and PTZ domains, may also contribute to interaction of signaling proteins (Harrison, 1996). The kinetics and magnitude of effector protein activation may also contribute to the action of growth factors (Qui and Green, 1992; Traverse et al., 1992; Marshall, 1995).

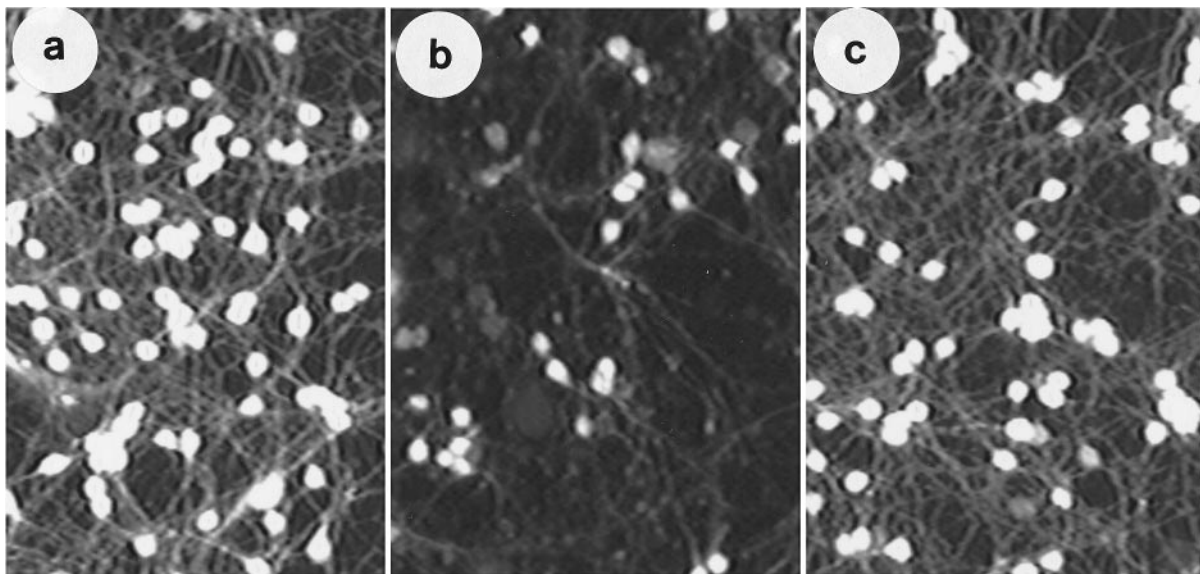
Although receptor properties, cellular context, and kinetics of activation may explain how growth factors elicit distinct biological actions, a question of fundamental importance is whether similar signaling components are used by different growth factors toward a common biological effect. One of the most striking properties of neurotrophic factors is their ability to maintain neuronal survival by inhibition of apoptosis. Most investigations of the signaling pathways involved in the prevention of apoptosis by neurotrophic

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**Figure 1.** IGF-1 can substitute for elevated  $K^+$  in the maintenance of neuronal survival. Granule neurons were cultured in BME supplemented with high  $K^+$  (25 mM KCl) and 10% FCS. After 7 d, the cultures were switched to serum-free BME (normally containing 5 mM KCl) supplemented with (a) 25 mM KCl, (b) no additives, and (c) IGF-1 (25 ng/ml). The figure shows fluorescent imaging of cultures by FDA staining at 24 hr after treatment. Note an increased number of fluorescent (viable) cells in elevated  $K^+$  and IGF-1 treated cultures (a, c) compared with culture switched to low  $K^+$  (b). For viability assays, an  $0.85 \text{ mm}^2$  field is randomly chosen from the culture dish and acquired using the NIH Image program. Stained cells in each field are then computed. In subsequent figures, phase-contrast micrographs that better display morphological appearance of the cultures have been used.

factors have been performed using the rat pheochromocytoma PC12 cell line (Greene and Tischler, 1976). When switched to serum-free medium, these cells undergo apoptosis (Batistatou and Greene, 1991; Mesner et al., 1992), which can be prevented by NGF or FGF (Rukenstein et al., 1991). Using the PC12 cell system, Yao and Cooper (1995) discovered recently that a critical component of the survival-promoting action of NGF is the SH2-containing enzyme phosphoinositide 3-kinase (PI 3-kinase).

PI 3-kinase is a heterodimer of a 85 kDa regulatory subunit and a 110 kDa catalytic subunit (Carpenter et al., 1990; Morgan et al., 1990; Escobedo et al., 1991; Hiles et al., 1992). The enzyme phosphorylates PI, PI-4P, and PI-4,5-P<sub>2</sub> on the D3 position of the inositol ring, leading to the formation of the lipids PI-3P; PI-3,4-P<sub>2</sub>; and PI-3,4,5-P<sub>3</sub>; respectively (for review, see Stephens et al., 1993; Kapeller and Cantley, 1994). Downstream target molecules of PI 3-kinase activation have not been definitively identified, although activation of certain serine/threonine kinases has been suggested (Toker et al., 1994; Burgering and Coffey, 1995; Franke et al., 1995). In addition to inhibiting apoptosis, activation of PI 3-kinase has been implicated in mitogenic signaling, metabolic processes (such as glucose uptake and superoxide production), membrane ruffling, and chemotaxis (for review, see Cantley et al., 1991 and Kapeller and Cantley, 1994).

Although PI 3-kinase is required for prevention of apoptosis in PC12 cells, the generality of this finding remains to be explored. Of particular interest is the question of whether PI 3-kinase is critical for the survival of normal neurons. Also unclear is whether neurotrophic factors other than NGF also use PI 3-kinase to support survival. To address these issues, we have used granule neurons cultured from postnatal rat cerebella. These cells constitute the most abundant neuronal population in the mammalian brain. When cultured from early postnatal rats, granule cells differentiate *in vitro*, acquiring several morphological, biochemical, and electrophysiological characteristics of mature neurons (Levi et al., 1984; Gallo et al.,

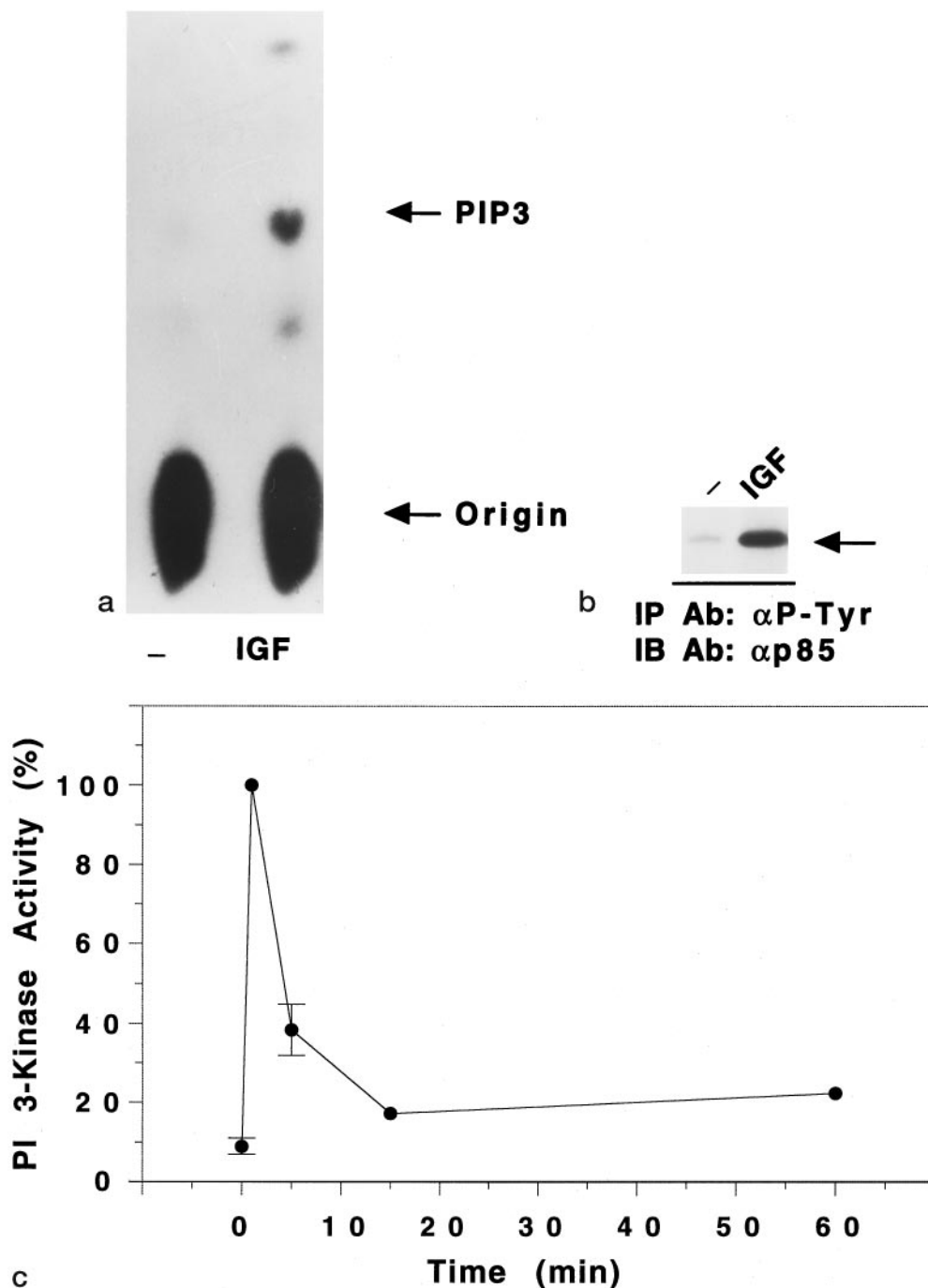
1987; Hockberger et al., 1987; Cull-Candy et al., 1988). An elevated level of  $K^+$  (25 mM) is necessary for the survival and differentiation of these neurons in culture (Lasher and Zaigou, 1972; Gallo et al., 1987). We have demonstrated previously that when switched from a culture medium containing high  $K^+$  (25 mM) to a lower, but physiological  $K^+$  medium (5 mM), fully differentiated granule neurons undergo apoptosis (D'Mello et al., 1993). Death by lowering of  $K^+$  can be prevented by IGF-1 but not by several other growth factors including FGF, NGF, BDNF, and neurotrophin-3 (D'Mello et al., 1993). Several pieces of evidence suggest that IGF-1 may be physiologically important for the development of granule neurons (Bondy et al., 1991; Gao et al., 1991; Calissano et al., 1993; Ye et al., 1996). Although mice-lacking functional IGF-1 have no dramatic deficit in granule cell number (Beck et al., 1995) presumably because of compensatory effects from related factors, other studies have shown that overexpression of IGF-1 increases proliferation and survival of granule cells (Ye et al., 1996). Beside its beneficial effect on granule cells, IGF-1 promotes the *in vitro* survival and neurite outgrowth of various sensory, sympathetic, cortical, and motor neurons (Aizenman and de Vellis, 1987; Caroni and Grandes, 1990; Svrzic and Schubert, 1990; Neff et al., 1993) (for review, see Bozyczko-Coyne et al., 1993).

We report that IGF-1 activates PI 3-kinase in cerebellar granule neurons and that the pharmacological inhibitors of PI 3-kinase prevent the survival-promoting action of IGF-1. In contrast, the elevation of extracellular  $K^+$  maintains survival of the same neurons by a PI 3-kinase-independent mechanism. Thus, cell survival and the block of apoptosis may occur by both PI 3-kinase-dependent and -independent pathways in granule neurons.

## MATERIALS AND METHODS

**Chemicals.** All chemicals were reagent grade or better. [<sup>32</sup>P]ATP (specific activity, 3000 Ci/mmol) was obtained from DuPont NEN (Boston, MA).

**Figure 2.** IGF-1 activates PI 3-kinase in granule neurons. *a*, Measurement of lipid kinase activity in anti-P-Tyr immunoprecipitates from cortical neurons. Cells were maintained overnight in serum-free medium containing 25 mM  $K^+$ , and then exposed (or not) to IGF-1 (25 ng/ml) for 1 min. PI 3-kinase was immunoprecipitated from the cleared lysates using anti-P-Tyr antibody (6.6  $\mu$ g/ml), and PI 3-kinase activity was measured using PI-4,5-P2 as a substrate. PI-3,4,5-P3 (*PIP3*), the main product of the lipid kinase assay, was identified using thin-layer chromatography. *b*, Immunoprecipitation of p85, the regulatory subunit of PI 3-kinase, from lysates of IGF-1-treated cells. The immunoprecipitated proteins used in the lipid kinase assay (*a*) were subjected to SDS-PAGE, transferred to nitrocellulose filters, and probed overnight with anti-p85 antibody (1:8000 dilution). The arrow on the right indicates the location of p85. The addition of IGF-1 produced a large increase in the amount of p85 that was immunoprecipitated using anti-P-Tyr antibody. *c*, Time course of PI 3-kinase activation by IGF-1. Granule neurons were exposed to IGF-1 (25 ng/ml) for various times between 1 and 60 min. Proteins were immunoprecipitated using anti-P-Tyr antibody, and PI 3-kinase activity was measured in the immunoprecipitates using PI-3,4-P2 as a substrate. The activities were normalized to the PI 3-kinase activity found at 1 min after IGF-1 treatment of cells cultured in serum-free medium containing 25 mM  $K^+$ . This time point always had the greatest activity. The values for the basal time (time 0), 1 min, and 5 min time points are the mean  $\pm$  SEM of three separate experiments. The values at 15 and 60 min were obtained from 1 and 2 of these same experiments, respectively.



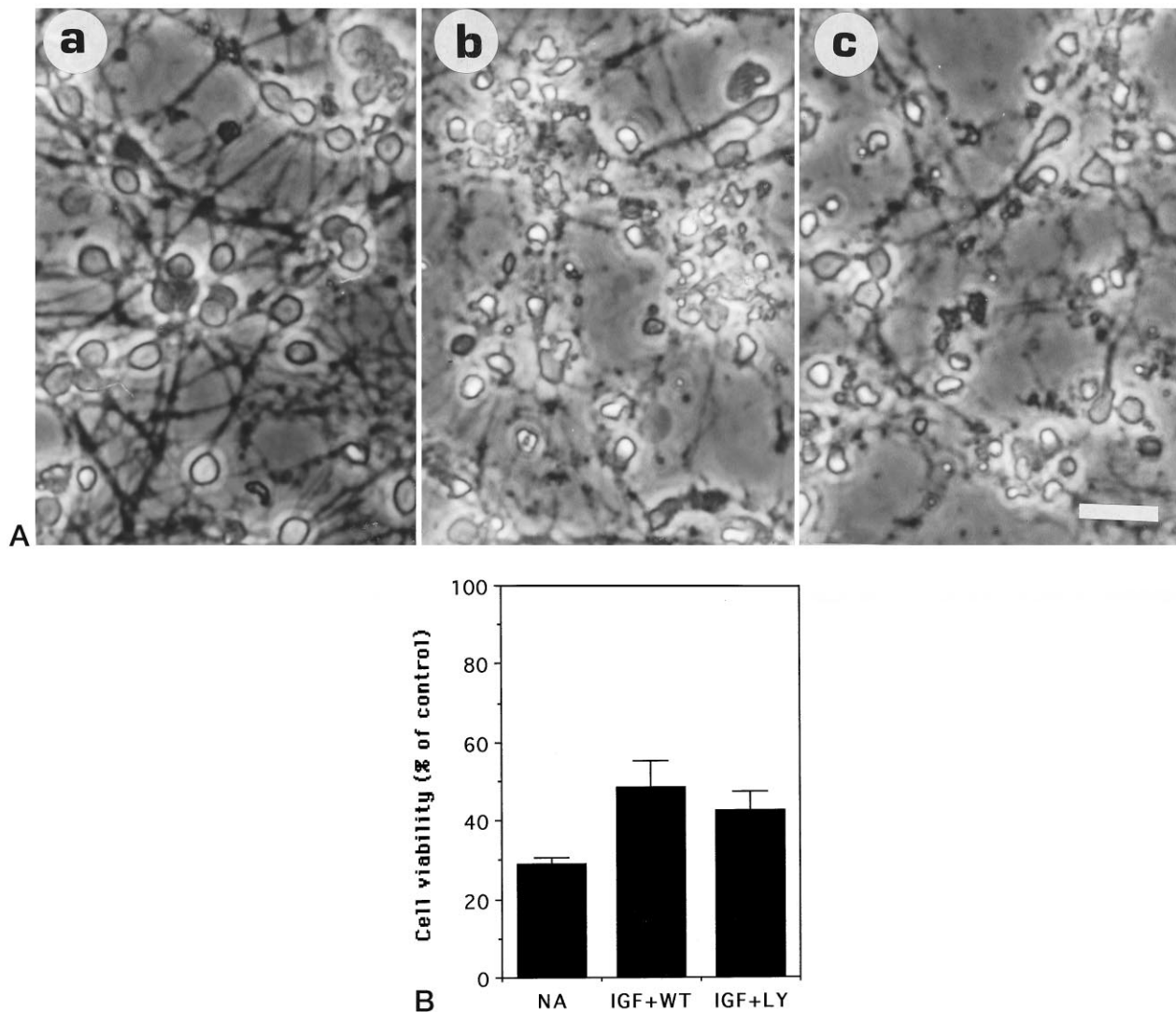
PI-4,5-P2 was obtained from Sigma (St. Louis, MO). Recombinant human IGF-1 and bFGF were purchased from Boehringer Mannheim Indianapolis, IN) and LY294002 from Biomol (Plymouth Meeting, PA). All other agents were obtained from Sigma. Recombinant mouse BDNF was a gift from Fidia Pharmaceuticals, Abano Terme, Italy.

**Antibodies.** Antibody to the p85 subunit of PI 3-kinase was raised in rabbits by Dr. Brian Schaufhausen (Tufts University, Boston, MA) and is commercially available from Upstate Biotechnology (Lake Placid, NY) (catalog no. 06-195), as is anti-phosphotyrosine antibody, clone 4G10 (05-321). An antibody made to insulin receptor substrate (IRS)-1 was generously donated by Dr. Ken Siddle (University of Cambridge, Cambridge, UK).

**Primary neuronal cultures.** Cultures enriched in granule neurons were obtained from dissociated cerebella of 8-d-old Wistar rats (Charles River, Wilmington, MA), as described by Thangnipon et al. (1983). After preparation, cells were plated in basal Eagle's medium (BME, Life

Technologies, Gaithersburg, MD) supplemented with 10% FCS, 25 mM KCl, 2 mM glutamine (Life Technologies), and 100  $\mu$ g/ml gentamycin (Life Technologies) on dishes (Nunc, Naperville, IL) coated with poly-L-lysine. Cells were plated at a density of  $3 \times 10^5$  per  $cm^2$  ( $2.5 \times 10^6$  cells/35 mm dish or  $25 \times 10^6/100$  mm dish). Cytosine arabinofuranoside (10  $\mu$ M) was added to the culture medium 18–22 hr after plating to prevent replication of non-neuronal cells. Immunocytochemical analysis of these primary cultures have shown that they contain >95% granule neurons (Thangnipon et al., 1983). Also, these cultures have been studied extensively and shown to possess the biochemical and electrophysiological characteristics of their counterparts *in vivo* (Levi et al., 1984; Gallo et al., 1987; Hockberger et al., 1987; Cull-Candy et al., 1988).

**Treatment of cultures.** Replacement of culture medium with serum-free medium was performed 7–8 d after plating as follows. Cells were washed once and maintained in serum-free BME culture medium supplemented with glutamine, gentamycin, and cytosine arabinofuranoside at the con-



**Figure 3.** Wortmannin and LY249002 inhibit the survival-promoting activity of IGF-1. Granule neurons maintained in high  $K^+$  and FCS (see Materials and Methods) were switched to serum-free medium containing (a) 25 ng/ml IGF-1, (b) 25 ng/ml IGF-1 + 50 nM wortmannin, and (c) 25 ng/ml IGF + 10  $\mu$ M LY249002. *A*, Morphological appearance at 24 hr. Scale bar, 20  $\mu$ m. *B*, Neuronal viability at 24 hr after treatment, measured by FDA staining, normalizing the data to the control (cells maintained in IGF-1) conditions. No additives, IGF-1, wortmannin, and LY249002 are designated as *NA*, *IGF*, *WT*, and *LY*, respectively. Error bars indicate mean  $\pm$  SD of data shown from three experiments performed in duplicate culture dishes. Two fields were examined from each dish.

centrations indicated above. The KCl concentration in serum-free media was 5 mM, unless specified otherwise. Factors and agents were added directly to the serum-free medium. Unless indicated otherwise, treatment with wortmannin or LY294002 included a pretreatment in which the drug was added to the cultures 15 min before exposure to stimuli.

**Neuronal survival.** Neuronal survival was quantified in cultures grown in 35 mm dishes by staining with 10  $\mu$ g/ml fluorescein diacetate (FDA, Sigma), as described (Jones and Senft, 1985). Viable cells are indicated by bright green on examination with a fluorescence microscope. Two fields from each dish were chosen randomly, and an area of 0.85 mm<sup>2</sup> was acquired using the NIH Image program. The number of surviving cells in the field were then computed. Typically, in healthy cultures (maintained in high  $K^+$  or IGF-1), the number of viable cells was 200–250/acquired field.

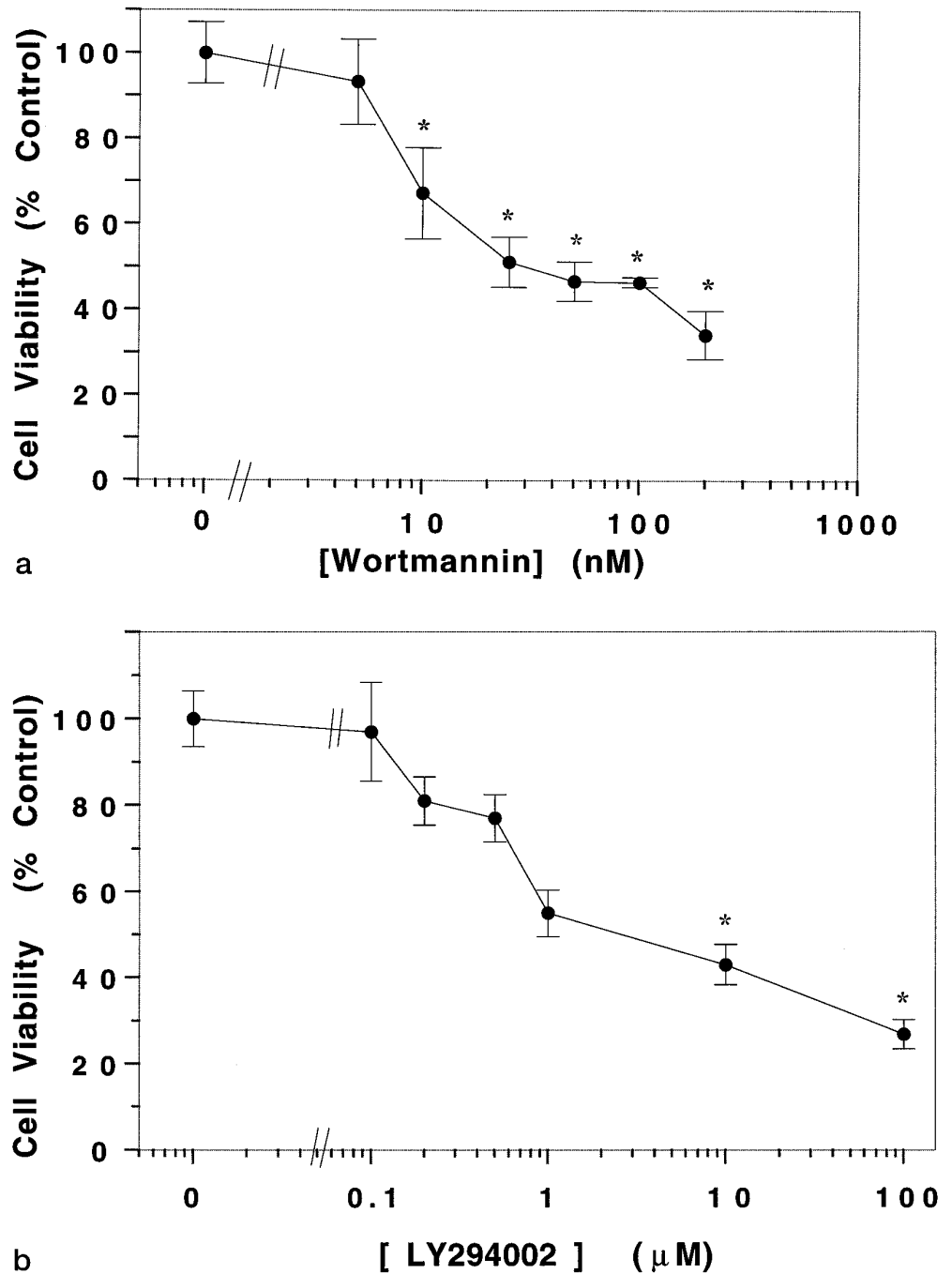
**DNA fragmentation analysis.** Fragmentation of DNA was analyzed as described previously (D'Mello et al., 1993). Equal numbers of cells ( $2.5 \times 10^6$ ) were plated and used for each set of treatments. After cell lysis and elimination of nuclei, soluble DNA was isolated. After treatment with RNase A (50 ng/ml) at 37°C for 30 min, the DNA was subjected to electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. Because the same number of cells were plated for each

treatment, the amount of soluble DNA that is recovered reflects the extent of genomic DNA damage.

**Measurement of PI 3-kinase activity.** The cells were exposed to growth factors for the indicated times at 37°C. Lysis of cells, immunoprecipitations, lipid kinase assays, and lipid extractions were performed as described previously (Soltoff et al., 1994). PI 3-kinase was immunoprecipitated using either anti-P-Tyr or anti-p85 antibodies, as specified. In experiments in which the *in vitro* wortmannin sensitivity of PI 3-kinase was analyzed, wortmannin was exposed to the immunoprecipitated PI 3-kinase for 15 min before addition of lipids and the kinase reaction mixture. Exogenous PI-4,5-P<sub>2</sub> was used as substrate to measure PI 3-kinase activity. The lipid products were separating using thin-layer chromatography and a solvent system composed of *N*-propanol: acetic acid (2 M) (65:35, vol:vol). The production of PI-3,4,5-P<sub>3</sub> was quantified using a Bio-Rad GS-363 Molecular Imager System.

**Identification of proteins by Western blot assays.** The electrophoresis of immunoprecipitated proteins and the Western blotting of proteins transferred from 7% polyacrylamide gels to nitrocellulose filters were performed as described previously (Soltoff et al., 1994). Proteins were visualized using a chemiluminescence system (Renaissance, Dupont NEN).

**Data.** Unless indicated otherwise, data are given as means + SEM, with



**Figure 4.** Dose response of wortmannin and LY294002 on granule neurons maintained in IGF-1. Cultures (7-d-old) were switched to medium containing IGF-1 (25 ng/ml) and (a) various concentrations of wortmannin and (b) various concentrations of LY294002. The figure shows neuronal viability at 24 hr after treatment. Error bars indicate mean  $\pm$  SD of data shown from three experiments performed in duplicate culture dishes. Two fields were examined from each dish. Asterisks indicate significant difference from control (no drug) treated cells ( $p < 0.05$ ).

the number of determinations ( $n$ ) representing separate experiments performed using single or duplicate samples.

For statistical significance, data were evaluated at a 0.05 level of significance with the Kruskal–Wallis ANOVA. Differences between individual groups were evaluated by the Newman–Keuls test.

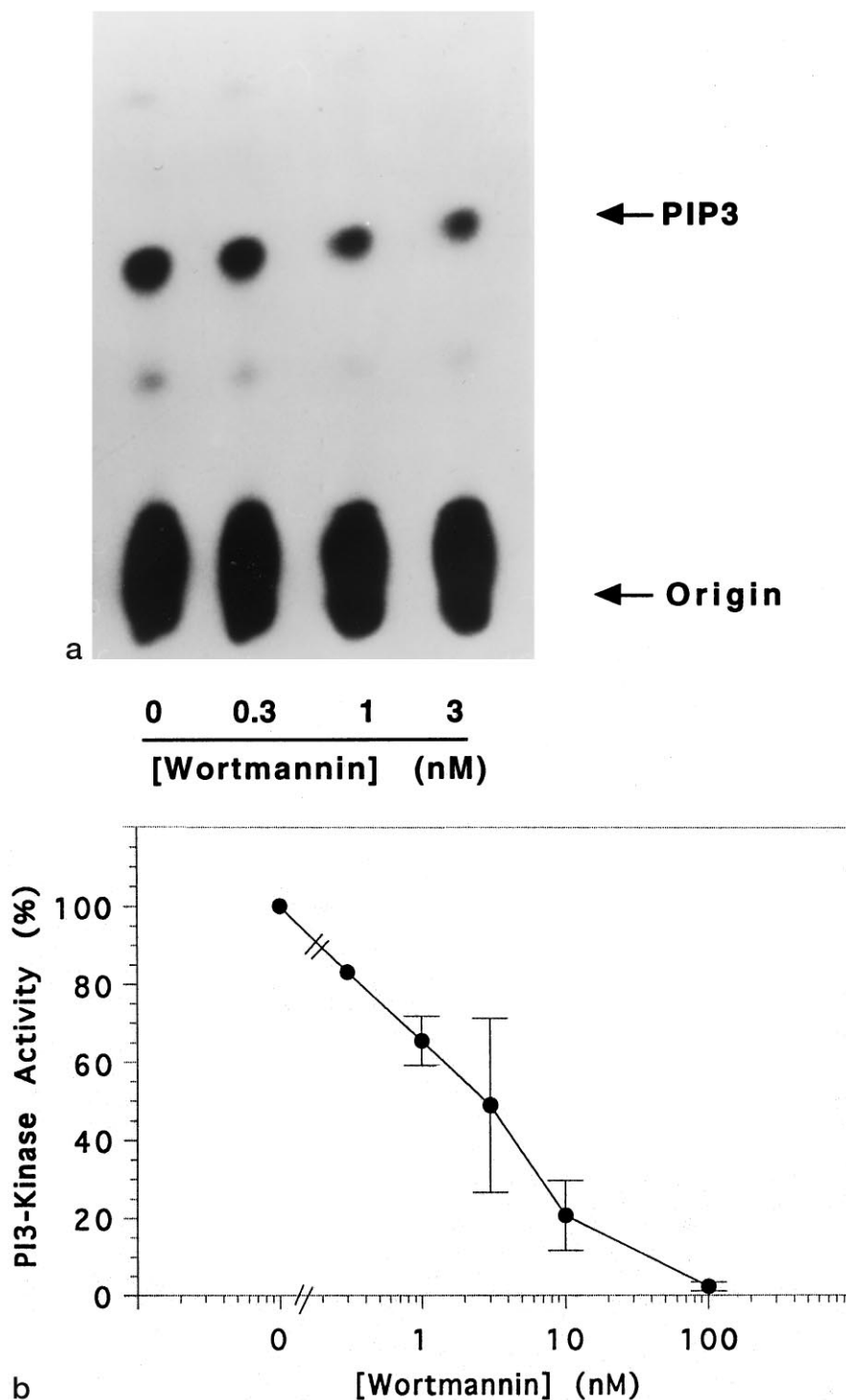
## RESULTS

A key issue in attempting to understand signal transduction pathways is whether similar intracellular signaling components are used by distinct growth factors toward a common biological effect. PI 3-kinase has been shown previously to be necessary for NGF-mediated survival of pheochromocytoma PC12 cells (Yao and Cooper, 1995). To examine whether this enzyme was also involved in promoting survival of normal neurons, we used cultures of rat cerebellar granule neurons.

### IGF-1 activates PI 3-kinase in granule neurons

Granule neuron cultures were cultured and maintained in medium supplemented with 25 mM KCl for 7–8 d, as described previously (D'Mello et al., 1993). The fully differentiated cultures were shifted to serum-free medium containing IGF-1. As we have reported previously (D'Mello et al., 1993) and as shown in Figure 1, IGF-1 (25 ng/ml) can efficiently substitute for high  $K^+$  in the maintenance of neuronal survival. In contrast, a majority of neurons in sister cultures lacking IGF-1 (low- $K^+$  medium, 5 mM KCl) die within 24 hr (Fig. 1) (D'Mello et al., 1993).

As a first step toward testing the hypothesis that maintenance of survival by IGF-1 involved PI 3-kinase, we examined whether PI 3-kinase was activated by IGF-1. Granule neuron cultures

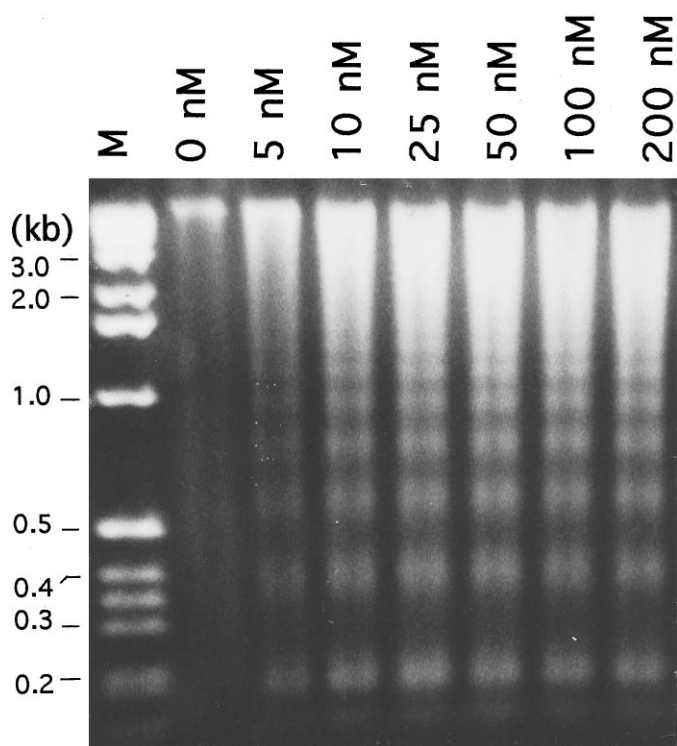


**Figure 5.** Wortmannin inhibits PI 3-kinase activity. *a*, PI 3-kinase activity was measured in lipid kinase assays using anti-p85 immunoprecipitates from lysates of granule cells maintained in high  $K^+$ . Immunoprecipitates were exposed (or not) to different concentrations of wortmannin, as indicated. PI-3,4,5-P<sub>3</sub>, the main product of the lipid kinase assay, was identified using thin-layer chromatography. *b*, The concentration dependence of the inhibition of PI 3-kinase activity by wortmannin. In each lipid kinase assay experiment, the data were normalized to the activity measured in the absence of wortmannin (0). Error bars indicate the mean values of three or four separate experiments, except for 0.3 nM ( $n = 1$ ).

were switched to medium containing IGF-1 (25 ng/ml). As shown in Figure 2*A*, treatment with IGF-1 resulted in a robust activation of PI 3-kinase within 1 min, as judged by the increased synthesis of PI-3,4,5-P<sub>3</sub> by PI 3-kinase immunoprecipitated using anti-P-Tyr antibody. In these experiments, IGF-1 (1 min) increased the PI 3-kinase activity to  $12.8 \pm 2.0$  ( $10 \times$ ) the basal activity. Consistent with this, there was a large increase in the association of PI 3-kinase (p85 subunit) with anti-P-Tyr antibody (Fig. 2*B*). The increase in PI 3-kinase activity was maximal at 1 min. Within 5 min, the degree of activation was

reduced, but it was sustained above basal levels for at least 1 hr (Fig. 2*C*).

In other experimental systems such as adipocytes and muscle, activation of PI 3-kinase by insulin and IGF-1 occurs via insulin receptor substrate (IRS) proteins (Sun et al., 1991, 1992). We examined whether IRS proteins were similarly involved in IGF-1 signaling in neurons. Consistent with findings from other cell systems, IGF-1 stimulated a large increase in PI 3-kinase activity in granule neurons that was immunoprecipitated using an anti-IRS-1 antibody. Using this antibody, the PI 3-kinase



**Figure 6.** Wortmannin-mediated death of neurons maintained in IGF-1 is accompanied by DNA fragmentation, a marker of apoptosis. DNA fragmentation analysis of cultures maintained in serum-free medium supplemented with IGF-1 (25 ng/ml) and treated with various concentrations of wortmannin. The wortmannin concentration (in nanomolars) is indicated at the top. Soluble DNA was isolated 18 hr after the switch, as described in Materials and Methods, treated with RNase A, and subjected to electrophoresis. Lane M contains a molecular weight marker, and numbers on the left represent lengths in kilobases (kb). The figure shows DNA visualized by ethidium bromide staining. Because the same number of cells was used for all concentrations, the amount of soluble DNA seen in each lane reflects the extent of DNA fragmentation. The small amount of fragmentation that is detectable in cultures treated with IGF-1 alone is attributable to the small amount of cell death observed when granule neurons are switched from high  $K^+$  to IGF-1-containing medium (D'Mello et al., 1993).

activity immunoprecipitated from cells treated with IGF-1 for 1 min was  $24.5 \pm 12.5$  (3) times the activity found in immunoprecipitations from untreated cells. Similar amounts of PI 3-kinase activity were immunoprecipitated from lysates of IGF-1-treated cells (1 min) using anti-P-Tyr and anti-IRS-1 antibodies; in experiments conducted in parallel, the activity immunoprecipitated using anti-IRS-1 antibody was  $1.5 \pm 0.4$  (3) times that immunoprecipitated using anti-P-Tyr antibody. In Western blot analysis, we observed that IGF-1 promoted an increase in the tyrosine phosphorylation of an  $\sim 170$  kDa protein that was immunoprecipitated using an anti-IRS-1 antibody (data not shown). In addition, IGF-1 promoted an increase in the tyrosine phosphorylation of an  $\sim 95$  kDa protein, the mass of the  $\beta$  subunit of the IGF-1 receptor (data not shown). These results suggest that autophosphorylation of the IGF-1 receptor results in binding and subsequent tyrosine phosphorylation of IRS-1, which in turn binds and activates PI 3-kinase.

#### PI 3-kinase activity is required for the prevention of neuronal apoptosis by IGF-1

We examined next whether the activation of PI 3-kinase was necessary for the IGF-1-dependent promotion of survival. To do

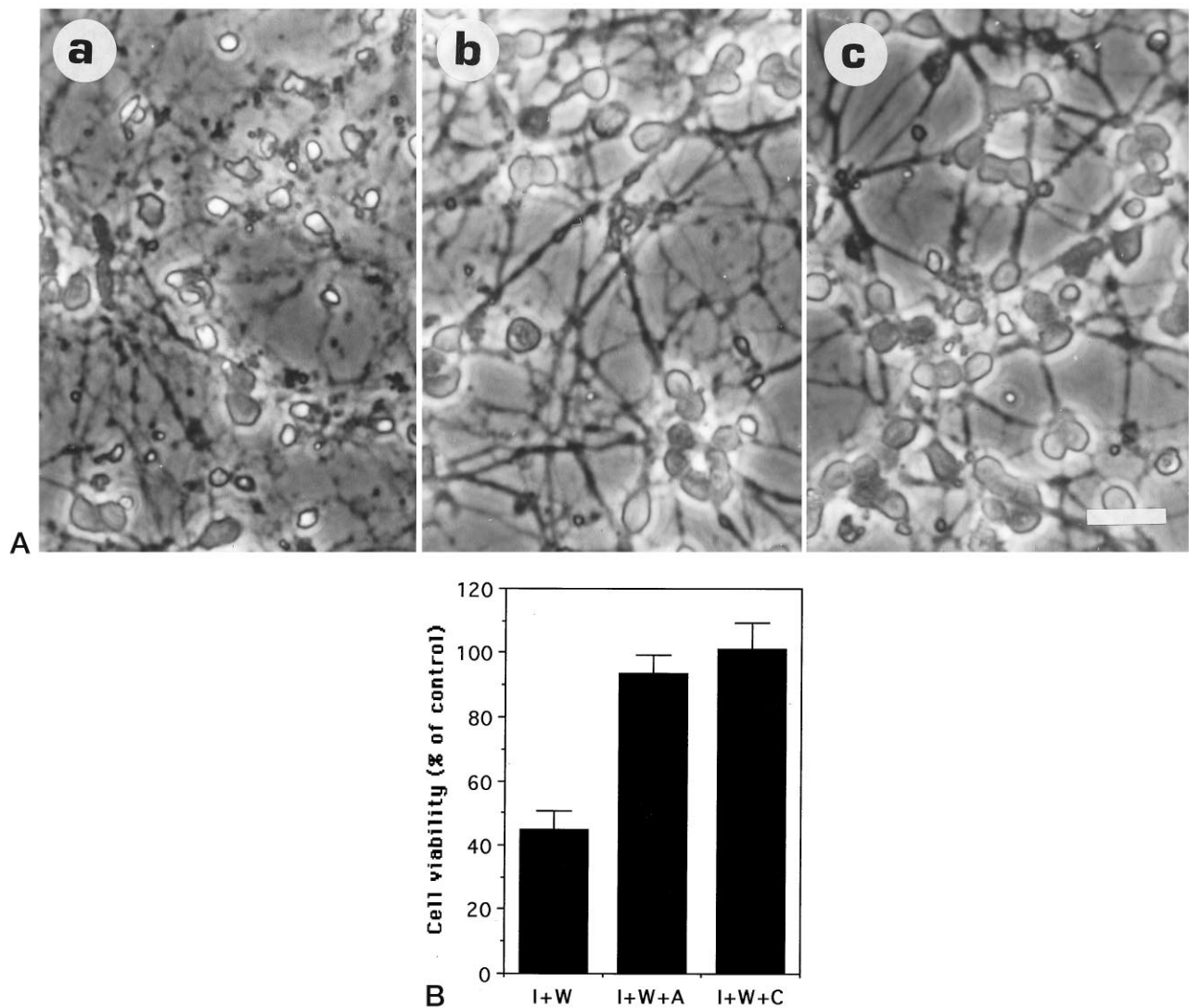
this, we used the fungal derivative wortmannin. Wortmannin has been shown previously to be a selective and potent inhibitor of PI 3-kinase in a number of experimental systems (Yano et al., 1993; Okada et al., 1994a,b; Ui et al., 1995). As shown in Figure 3, A and B, when cultures were co-treated with IGF-1 and wortmannin (50 nM), neuronal viability was  $48.5 \pm 6.5\%$  (6) of that observed with IGF-1 alone. The sharp reduction in viability with wortmannin is consistent with an involvement of PI 3-kinase in IGF-mediated inhibition of neuronal death (Fig. 3B). Recent reports from some laboratories have shown that at higher concentrations, wortmannin may also have inhibitory effects on other cellular targets. To verify our finding that PI 3-kinase was involved in IGF-1-mediated cell survival, we used another specific inhibitor of PI 3-kinase, LY 294002 (Sanchez-Margalet et al., 1994; Vlahos et al., 1994). As shown in Figure 3, A and B, and as observed with wortmannin, treatment of cultures maintained in IGF-1 with  $10 \mu\text{M}$  LY294002 caused extensive cell death [ $42.5 \pm 4.7\%$  (6) of cultures treated with IGF-1 alone].

A dose-response analysis of the effect of wortmannin revealed that a statistically significant effect on neuronal viability was detectable at doses  $\geq 10$  nM (Fig. 4A). The inhibitory effect of wortmannin on other enzymes occurs at micromolar concentrations (Okada et al., 1994a,b). The ability of wortmannin to inhibit neuronal survival at nanomolar doses, therefore, suggests that its effect is attributable to its established inhibitory action on PI 3-kinase. The concentration dependence of LY294002 on neuronal viability is shown in Figure 4B. Cell viability was significantly affected at concentrations of LY294002  $\geq 10 \mu\text{M}$ , a concentration similar to that determined previously to be required for its inhibition of PI 3-kinase (Sanchez-Margalet et al., 1994; Vlahos et al., 1994).

To demonstrate that wortmannin blocked PI 3-kinase activity, we performed *in vitro* assays of the enzyme immunoprecipitated from neurons using an anti-p85 antibody. PI 3-kinase activity was reduced in a concentration-dependent manner by wortmannin (Fig. 5). Under these conditions, the  $IC_{50}$  value was  $\sim 3$  nM wortmannin. When cells were treated *in vivo* with wortmannin (100 nM), the IGF-1-promoted PI 3-kinase activity was reduced by  $61.7 \pm 4.9\%$  (4). The concentrations of wortmannin that inhibited PI 3-kinase, therefore, were similar to those required to inhibit survival of neuronal cultures by IGF-1. Taken together, these results indicate that the survival of granule neurons by IGF-1 requires the activation of PI 3-kinase.

#### Inhibition of PI 3-kinase is accompanied by DNA fragmentation, a hallmark of apoptosis

We have shown previously that IGF-1 maintains granule neuron survival by blocking apoptosis (D'Mello et al., 1993). Blockade of IGF-1 signaling, therefore, would be expected to induce apoptosis. We proceeded to confirm that inhibition of PI 3-kinase, an essential component of IGF-1 signaling, induced death via apoptosis. A highly reliable biochemical marker of apoptotic death is the cleavage of genomic DNA into nonrandom, oligonucleosomal-size fragments. As shown in Figure 6, DNA isolated from neuronal cultures co-treated with IGF-1 and wortmannin displayed the characteristic "DNA ladder" of apoptosis. Significant fragmentation was observed at doses  $\geq 10$  nM, the same concentrations of wortmannin as those causing death. Not unexpectedly, virtually no DNA fragmentation was observed in IGF-1 treated cells in the absence of wortmannin. The similarity in the morphological appearance of cells dying as a result of lowering of  $K^+$  in serum-free medium



**Figure 7.** The transcriptional inhibitor actinomycin D and the translational inhibitor cycloheximide prevent wortmannin-promoted death. Neuronal cultures (7-d-old) were switched to serum-free medium containing (a) 25 ng/ml IGF-1 + 50 nM wortmannin (*I + W*), (b) 25 ng/ml IGF-1 + 50 nM wortmannin + 2 μg/ml actinomycin D (*I + W + A*), and (c) 25 ng/ml IGF-1 + 50 nM wortmannin + 10 μg/ml cycloheximide (*I + W + C*). Data shown were obtained from two experiments performed in duplicate culture dishes. Two fields were examined from each dish. *A*, Morphological appearance at 24 hr after treatment. Scale bar, 20 μm. *B*, Neuronal viability measured at the same time point as in *A*. The data are normalized to that obtained in the presence of 25 ng/ml IGF-1 without inhibitors.

to those exposed to wortmannin in IGF-1-containing medium suggests that wortmannin did not have a general necrotic effect on the cells.

#### Wortmannin-mediated death of granule neurons can be prevented by inhibitors of gene expression

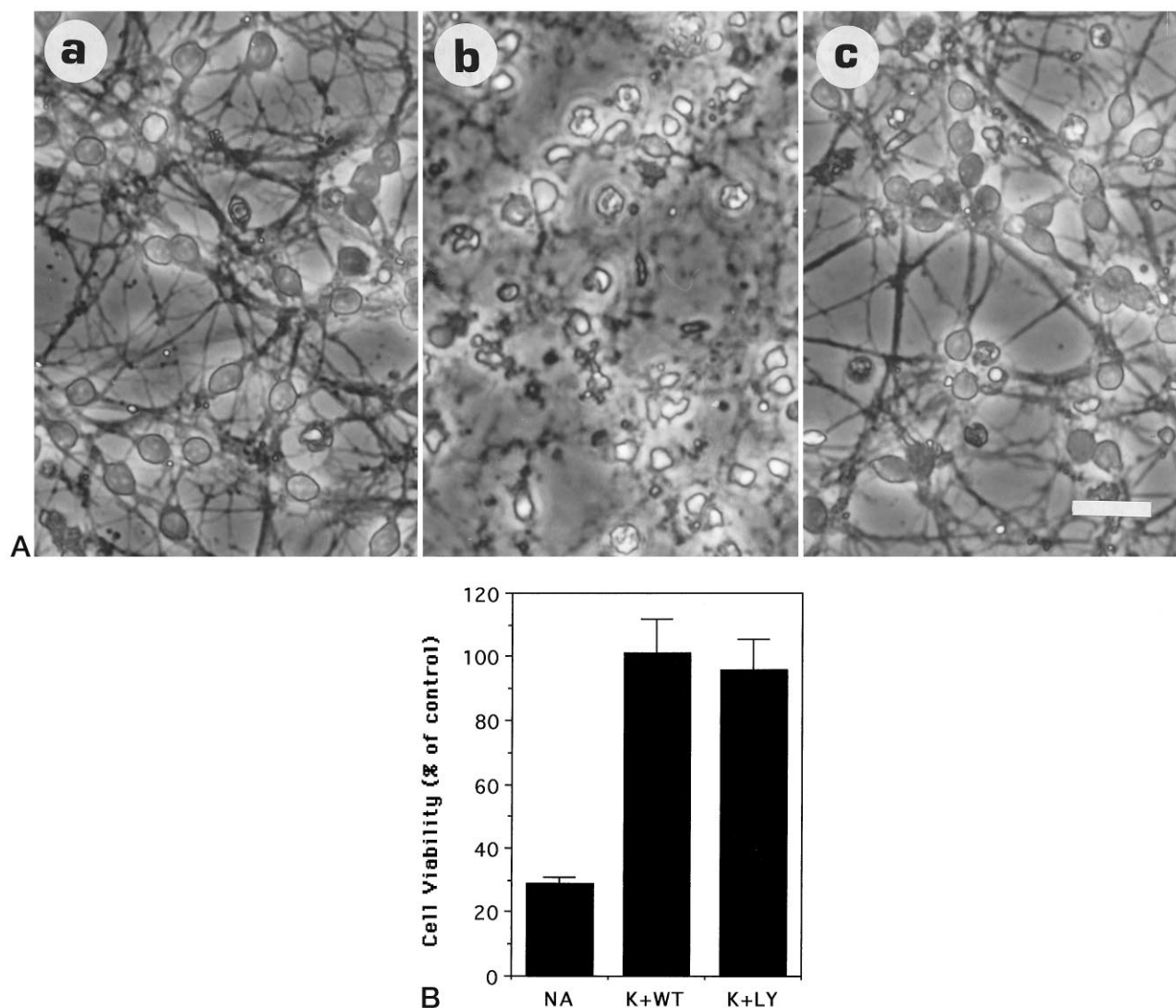
In most cell culture paradigms, death by apoptosis can be delayed by inhibitors of RNA and protein synthesis. These findings have led to the hypothesis that apoptosis is dependent on the synthesis of new gene products and that trophic factors maintain survival by suppressing production of these potentially lethal molecules. We have shown previously that macromolecule synthesis inhibitors can prevent apoptosis of granule neurons caused by lowering of extracellular  $K^+$ . To examine whether wortmannin-induced death could also be blocked by inhibitors of gene expression, we used the transcriptional inhibitor actinomycin D and the translational inhibitor cycloheximide. As shown in Figure 7, *A* and *B*, both of

these inhibitors of gene expression prevented wortmannin-mediated neuronal death.

#### Elevated $K^+$ mediated inhibition of apoptosis is not dependent on PI 3-kinase

A number of polypeptide growth factors have been shown to be capable of activating PI 3-kinase in other cell types (Stephens et al., 1993; Kapeller and Cantley, 1994). Furthermore, activation of PI 3-kinase is known to be associated with a variety of biological effects such as membrane ruffling, chemotaxis, cell survival, and mitogenesis. To determine whether PI-3 kinase activation in granule neurons was in any way selective for the promotion of survival, we examined whether activation of receptor tyrosine kinases other than the IGF-1 receptor also activated PI 3-kinase. Neither bFGF nor BDNF activated PI 3-kinase (S. Soltoff and S. D'Mello, unpublished observations), although the receptors for both these growth factors are expressed in granule neurons (Bondy, 1991;





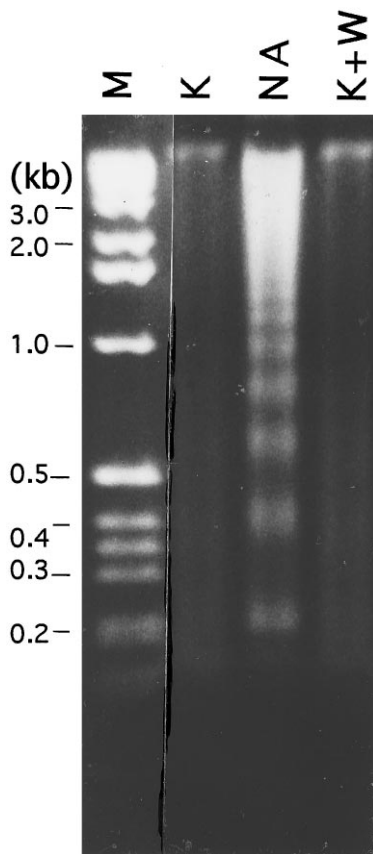
**Figure 8.** Wortmannin and LY249002 do not inhibit survival promoted by high  $K^+$ . Granule neurons were switched to serum-free medium containing (a) 25 mM KCl, (b) no additives (5 mM  $K^+$ ), and (c) 25 mM KCl + 50 nM wortmannin. *A*, Morphological appearance of neuronal cultures at 24 hr after treatment. Scale bar, 20  $\mu$ m. *B*, Quantification of cell survival as measured by FDA staining. No additives, 25 mM KCl, 50 nM wortmannin, and 10  $\mu$ M LY294002 are designated as *NA*, *K*, *WT*, and *LY*, respectively. Error bars indicate mean  $\pm$  SD of data from three experiments performed in duplicate culture dishes. Two fields were examined from each dish. The data are normalized to that obtained in the presence of 25 mM KCl without inhibitors. Similar results were observed in other experiments.

Klein et al., 1993; Ringstedt et al., 1993). As we have reported previously (D'Mello et al., 1993), neither bFGF nor BDNF has a significant survival effect on these neurons in our cell culture paradigm. This finding shows that polypeptide factors that do not promote survival fail to activate PI 3-kinase, suggesting that activation of this enzyme may be associated with the promotion of survival by growth factors such as IGF-1 and NGF (in PC12 cells).

The above observations raised the possibility that PI 3-kinase activation was critical and essential for survival of granule neurons. To address this issue, we examined whether PI 3-kinase was used by survival-promoting agents other than IGF-1. As described previously, one of the most efficient promoters of granule cell survival is an elevated level of extracellular  $K^+$  (Lasher and Zaigon, 1972; Gallo et al., 1987; D'Mello et al., 1993; Yan et al., 1994). To examine whether PI 3-kinase activity was necessary for the survival-promoting action of elevated  $K^+$ , granule cell cultures were switched to serum-free medium containing high  $K^+$  (25 mM

KCl) and wortmannin. In contrast to its lethal effect on cultures maintained in IGF-1, wortmannin had no effect on the ability of elevated  $K^+$  to support survival. As shown in Figure 8, *A* and *B*, viability of cultures shifted to serum-free medium containing high  $K^+$  alone were comparable with those co-treated with high  $K^+$  and wortmannin. As observed with wortmannin, the survival-promoting action of  $K^+$  was also not affected by LY294002 (Fig. 8*B*). We examined next whether DNA fragmentation occurred in neurons co-treated with elevated  $K^+$  and wortmannin. As shown in Figure 9, no fragmentation of DNA was detectable in cells co-treated with  $K^+$  and wortmannin, even when the drug was used at relatively high concentrations (200 nM). This result suggests that wortmannin was not toxic to the cells but acted in a specific manner to induce apoptosis by blocking IGF-1-mediated activation of PI 3-kinase.

To confirm that the survival pathway of  $K^+$  did not involve PI 3-kinase, we analyzed the activity of this enzyme after treatment



**Figure 9.** Fragmentation of DNA cannot be detected in cultures co-treated with wortmannin and KCl. DNA fragmentation analysis of cultures maintained in high KCl (25 mM) and treated with 200 nM wortmannin. Soluble DNA was isolated 18 hr after the switch, as described in Materials and Methods, treated with RNase A, and subjected to electrophoresis. Lane M contains a molecular weight marker, and numbers on the left represent lengths in kilobases (kb). The figure shows DNA visualized by ethidium bromide staining.

of cells with high  $K^+$ . In this experiment, neuronal cultures were switched to serum-free medium (5 mM  $K^+$ ) containing no additives for 4 hr. High  $K^+$  or IGF-1 was added to separate cultures, and PI 3-kinase activity was measured. As shown in Figure 10, the addition of IGF-1 caused a dramatic increase in PI 3-kinase activity. On the other hand, high  $K^+$  had no significant effect on enzyme activity. Taken along with the lack of effect of wortmannin and LY294002 on various survival parameters, these results suggest that in contrast to the pathway activated by IGF-1,  $K^+$ -mediated survival does not require PI 3-kinase activity. Therefore, granule cell survival may be mediated by a PI 3-kinase-dependent as well as a PI 3-kinase-independent signaling pathway.

## DISCUSSION

In this report, we describe two new findings. First, we present data consistent with a requirement for PI 3-kinase in the survival-promoting action of IGF-1 in normal neurons. This finding supports the possibility that common signaling components are activated by NGF (in PC12 cells) and IGF-1 (in cerebellar granule neurons) toward the maintenance of cell survival. Secondly, we show that although necessary for IGF-1 signaling, PI 3-kinase activity is not required for the promotion of survival by elevated  $K^+$ , implying that more than one pathway can mediate neuronal survival.

## Neuronal survival by IGF-1 is dependent on PI 3-kinase activity

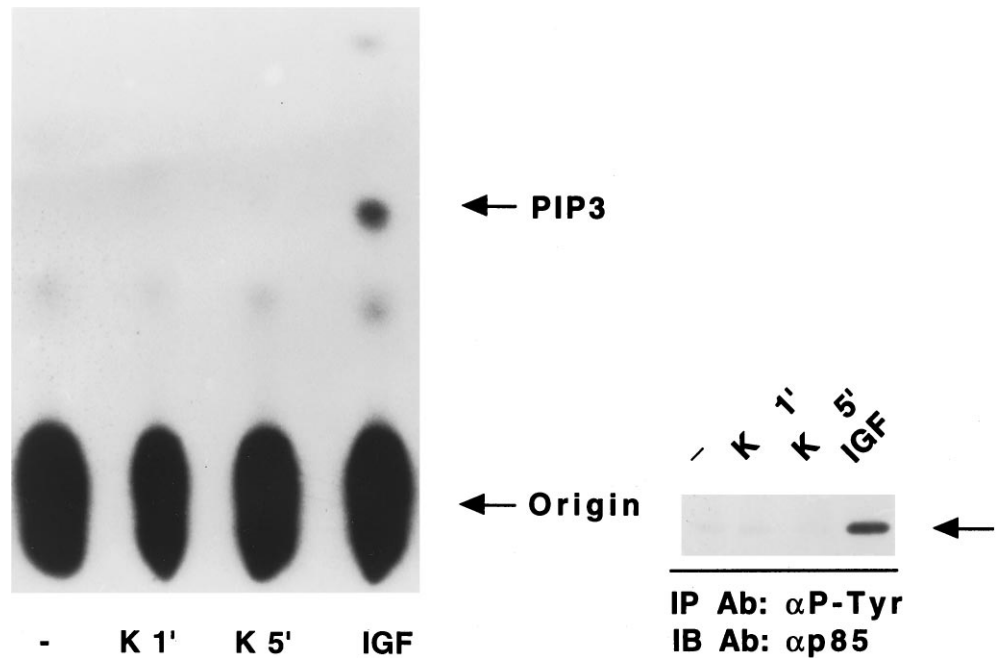
Regulation of neuronal survival is controlled by a complex array of intracellular signaling pathways. Most of these studies have been limited to the actions of NGF in PC12 cells. In addition to that of NGF, survival of a variety of neuronal types can be maintained by IGF-1 (Aizenman and de Vellis, 1987; Caroni and Grandes, 1990; Svrzic and Schubert, 1990; D'Mello et al., 1993; Neff et al., 1993) (for review, see Bozyczko-Coyne et al., 1993). In contrast to the actions of NGF, however, little is known about how IGF-1 might exert its neurotrophic actions. Results from non-neuronal systems show that a major target of the IGF-1 receptor tyrosine kinase is IRS-1 (Lamphere and Lienhard, 1992; Myers et al., 1993; Hernandez-Sanchez et al., 1995) and, more recently, IRS-2. Exposure of cells to IGF-1 results in the tyrosine phosphorylation of IRS-1 and the subsequent recruitment of multiple SH2-containing signaling proteins, including PI 3-kinase, to specific binding motifs on IRS-1 (Myers et al., 1993; Kotani et al., 1994).

In this study, we investigated the role of PI 3-kinase in IGF-1 mediated survival of primary neurons. We report that PI 3-kinase may be a key component of the survival-promoting pathway activated by IGF-1 in granule neurons. Although definitive proof of the necessity for PI 3-kinase in neuronal survival may require experiments such as the overexpression of dominant-negative forms of the enzyme, we present compelling evidence that this is likely to be the case. IGF-1 produces a large increase in PI 3-kinase activity in granule cells, and a sustained activation continues up to 1 hr. Wortmannin, a highly selective inhibitor of PI 3-kinase ( $IC_{50}$ ,  $\sim 3$  nM *in vitro*), blocks the *in vivo* activation of PI 3-kinase by IGF-1. Treatment with wortmannin also significantly blocks the survival-promoting action of IGF-1 and induces apoptosis at concentrations  $\geq 10$  nM. It deserves mention that in some studies, wortmannin has been shown to inhibit other enzymes including myosin light-chain kinase, phosphatidylinositol 4-kinase, and phospholipase D. These effects, however, are observed at micromolar concentrations of this drug (Okada et al., 1994a,b). Additional support for the involvement of PI 3-kinase in neuronal survival comes from our finding that LY294002, another inhibitor of PI 3-kinase that is chemically unrelated to wortmannin, also blocks the survival action of IGF-1. Therefore, it is likely that IGF-1 maintains the survival of granule cells via a PI 3-kinase-dependent pathway. The increase in PI 3-kinase activity of IGF-1-treated cells was observed in both anti-P-Tyr immunoprecipitates and anti-IRS-1 immunoprecipitates. Thus, our data suggest that IGF-1 promotes the association of IRS-1 with PI 3-kinase in granule neurons.

## Inhibition of PI 3-kinase in neurons maintained in IGF-1 activates an apoptotic pathway

DNA fragmentation, a characteristic feature of apoptotic death, is clearly detectable in wortmannin-treated neurons primed to die (Fig. 6). More important, fragmentation of DNA by wortmannin occurs at doses similar to that which causes cell death. An interesting feature of wortmannin-mediated cell death is that it can be prevented by transcriptional and translational inhibitors. The ability of transcriptional and translational inhibitors to prevent apoptosis is believed to be attributable to suppression of expression of specific genes (killer genes) that are required for the death process. Assuming that the effect of wortmannin on granule neurons maintained in IGF-1 is attributable to its inhibition of PI 3-kinase, it is possible that in healthy neurons, PI 3-kinase activation is

**Figure 10.** IGF-1 but not  $K^+$  increases PI 3-kinase activity in serum-deprived neurons. Cells were serum-starved and maintained overnight in medium containing 25 mM  $K^+$  and then switched for 4 hr to a serum-free medium containing 5 mM  $K^+$ . Cells were then exposed acutely to 25 mM  $K^+$  for 1 or 5 min ( $K 1'$  or  $K 5'$ ) or IGF-1 (25 ng/ml) for 1 min. PI 3-kinase was immunoprecipitated from the cleared lysates using anti-P-Tyr antibody (6.6  $\mu$ g/ml). *Left panel*, PI 3-kinase activity was measured in a lipid kinase assay using PI-4,5-P<sub>2</sub> as a substrate. PI-3,4,5-P<sub>3</sub> (PIP<sub>3</sub>), the main product of the lipid kinase assay, was separated using thin-layer chromatography. *Right panel*, The immunoprecipitated proteins used in the lipid kinase assay were subjected to SDS-PAGE, transferred to nitrocellulose filters, and probed overnight with anti-p85 antibody (1:8000 dilution). The arrow on the right indicates the location of p85. IGF-1, but not  $K^+$ , produced an increase in the association of p85 with the anti-P-Tyr antibody. Similar results to those shown in top and bottom panels were obtained in at least two other additional experiments. See Materials and Methods for additional details.



upstream of the suppression of killer genes. Blockade of PI 3-kinase activity, therefore, may relieve this suppression, resulting in the expression of killer genes that ultimately causes cell death.

Interestingly, both the IGF-1 receptor and the PI 3-kinase mRNAs are expressed in the cerebellar granule cell layer of the rodent brain (Bondy, 1991; Folli et al., 1994; Ito et al., 1995), suggesting that the trophic actions of IGF-1 *in vivo* may also be mediated through PI 3-kinase activation. It is likely that this activation of PI-3 occurs via IRS-1. Intriguingly, in a recent study by Folli et al. (1994), IRS-1 immunoreactivity was not detectable in the internal granule layer of the rat cerebellum. Expression of IRS-1 in granule neurons *in vivo* may be low and possibly below the detection level of immunocytochemical techniques. Alternatively, IGF-1 signaling might occur via IRS-2, for which a detailed localization study has not been reported. In this regard, the anti-IRS-1 antibody used in this study was generated to a peptide sequence to IRS-1 before the initial report of IRS-2, and the antibody has not been examined for its ability to recognize IRS-2.

The necessity for PI 3-kinase in survival promotion by both NGF and IGF-1 raises the possibility that although acting on distinct tyrosine kinase receptors, the two factors might activate common intracellular signaling pathways. Other polypeptide factors acting through tyrosine kinase receptors may also use the same signaling components (such as PI 3-kinase) to maintain survival. Support for this idea comes from the finding that PDGF-mediated survival of PC12 cells may also involve PI 3-kinase. Apoptosis of PC12 cells could be prevented by platelet-derived growth factor (PDGF) in cell lines engineered to express the wild-type PDGF receptor, but not in cells expressing a mutated PDGF receptor unable to bind PI 3-kinase (Yao and Cooper, 1995).

#### *PI 3-kinase is not required for survival promoted by elevated $K^+$*

We examined whether PI 3-kinase was a central and essential component of the biochemical machinery used by granule neurons for survival. These experiments were prompted by our ob-

servation that factors (bFGF and BDNF) that did not affect survival of granule cells in this paradigm also did not activate PI 3-kinase. Interestingly, neither wortmannin nor LY294002 affected the ability of high  $K^+$  to maintain survival, even when used at high doses. DNA fragmentation was also not detected in neurons maintained in elevated  $K^+$  and treated with wortmannin. In addition, PI 3-kinase was not activated when cells were shifted from serum-free medium containing the normal  $K^+$  concentration (5 mM) to that which contained the high concentration (25 mM). It appears, therefore, that high  $K^+$  maintains neuronal survival by a PI 3-kinase-independent pathway that is distinct from that activated by IGF-1. It is generally believed that cell survival is regulated by a common biochemical pathway. If this is in fact the case, our results suggest that PI 3-kinase activation in the IGF-1 pathway lies upstream of the point at which the high- $K^+$  and IGF-1 pathways converge.

Results from a previous study by Galli et al. (1995) have also provided evidence that IGF-1 and high  $K^+$  act through distinct mechanisms in cerebellar granule neurons. These authors showed that although influx of  $Ca^{2+}$  through voltage-gated channels is involved in the survival-promoting pathway of elevated  $K^+$ , it does not occur with IGF-1. Like IGF-1, the cyclic AMP elevating agent forskolin, which is also capable of maintaining survival of granule neurons in culture (D'Mello et al., 1993), did not stimulate  $Ca^{2+}$  influx (Galli et al., 1995). Although this result raises the possibility that IGF-1 and forskolin may act by a common mechanism that is not associated with  $Ca^{2+}$  influx, we have observed recently that forskolin-mediated survival is not inhibited by wortmannin (K. Borodezt and S. D'Mello, unpublished observations). Hence, based on influx of  $Ca^{2+}$  and PI 3-kinase-dependence, it is likely that  $K^+$ , IGF-1, and cAMP act by three distinct mechanisms.

In addition to PI 3-kinase, mitogen-activated protein kinase family members have been shown to both positively and negatively regulate cell death. Xia et al. (1995) showed recently that in NGF-differentiated PC12 cells, which undergo apoptosis at NGF deprivation, the extracellular signal-regulated kinase was involved

in maintaining survival, whereas activation of c-jun NH<sub>2</sub>-terminal protein kinase (JNK) induced apoptosis. Earlier studies have shown that the expression of c-jun, a target of JNK, is induced in cultured sympathetic neurons during apoptosis and is involved in the mediation of cell death (Estus et al., 1994; Ham et al., 1995). It remains to be tested whether extracellular signal-regulated kinase and JNK are involved in the survival-promoting action of IGF-1 in granule neurons. Another molecule that might be involved in PI-3 kinase-mediated survival is the Akt kinase. Akt has been shown previously to be a target of PDGF-activated PI 3-kinase (Burgering and Coffey, 1995; Franke et al., 1995).

Massive degeneration of cerebellar Purkinje neurons as well as granule cells occurs in ataxia telangiectasia (AT), a human genetic disorder afflicting children. Recently, the gene encoding the mutated protein in AT was identified and found to structurally resemble the PI 3-kinase gene (Lavin et al., 1995; Savitsky et al., 1995). It is not yet known whether the normal AT gene does, in fact, encode an enzyme with PI 3-kinase activity or whether patients with AT have a deficiency in PI-3 kinase activity. Assuming this to be the case, however, it is tempting to speculate that finding out how elevated K<sup>+</sup> promotes survival independently of PI 3-kinase could lead to the development of therapeutic approaches toward preventing the neuronal loss that occurs in AT.

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