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Inhibition of Phosphatidylinositol 3-Kinase Activity Blocks Depolarization- and Insulin-like Growth Factor I-mediated Survival of Cerebellar Granule Cells*

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Depolarizing concentrations of potassium promote the survival of many neuronal cell types including cerebellar granule cells. To begin to understand the intracellular mediators of neuronal survival, we have tested whether the survival-promoting effect of potassium depolarization on cerebellar granule cells is dependent on either mitogen-activated protein (MAP) kinase or phosphatidylinositol 3-kinase (PI-3-K) activity. In 7-day cerebellar granule cell cultures, potassium depolarization activated both MAP kinase and PI-3-K. Preventing the activation of MAP kinase with the MEK1 inhibitor PD98059 did not affect potassium saving. In contrast, the survival-promoting effect of 25 mM potassium was negated by the addition of 30 μM LY 294002 or 1 μM wortmannin, two distinct inhibitors of PI-3-K. The cell death induced by PI-3-K inhibition was indistinguishable from the cell death caused by potassium deprivation; LY 294002-induced death included nuclear condensation, was blocked by cycloheximide, and had the same time course as potassium deprivation-induced cell death. Cerebellar granule cells can also be maintained in serum-free medium containing either 100 ng/ml insulin-like growth factor I (IGF-I) or 800 μM cAMP. PI-3-K inhibition completely blocked the survival-promoting activity of IGF-I, but had no effect on cAMP-mediated survival. These data indicate that the survival-promoting effects of depolarization and IGF-I, but not cAMP, require PI-3-K activity.

Cell death is a widespread event during development that, in the nervous system, is thought to match the size of neuronal populations with their targets (1). One mechanism for determining which neurons survive and which undergo programmed cell death is limiting the amount of neurotrophic factors (1). Another important mechanism for determining survival is electrical activity; removal of afferent input or pharmacological blockade of electrical activity causes the death of some types of developing neurons (2–6). *In vitro*, mimicking electrical activity with depolarizing concentrations of extracellular potassium promotes the survival of many types of neurons (for review, see Ref. 7). Depolarization leads to the sustained activation of voltage-gated calcium channels and subsequent elevation of intracellular calcium levels. The increase in intracellular calcium is important for survival because blockade of L-type calcium channels blocks K^+-mediated survival (8–14), yet the intracellular pathways that mediate this survival are unknown.

Dissociated cerebellar granule cells from early postnatal rats can be maintained in serum-containing medium by raising the extracellular potassium concentration to 25 mM (15). Dissociated granule cells develop characteristics of mature cerebellar granule cells *in vivo* including an extensive neuritic network, expression of excitatory amino acid receptors, and production and release of L-glutamate (16). Removal of both potassium and serum from the culture medium triggers a cell death that is morphologically apoptotic, accompanied by DNA fragmentation, and dependent on macromolecular synthesis (17). This programmed cell death (PCD)[1] presumably mimics the naturally occurring death of 20–30% of granule cells (18), thought to be important for matching the number of granule cells with Purkinje cells, that occurs during the third through fifth postnatal weeks (19, 20).

One possible mechanism by which depolarization could promote survival is by activating survival-promoting signaling pathways similar to those activated by neurotrophic factors. Tyrosine kinase growth factor receptors activate several intracellular signaling pathways. Of these, the MAP kinase and phosphatidylinositol 3-kinase (PI-3-K) pathways have been implicated in survival. In PC12 cells, overexpression of a constitutively active form of MEK1 (MAP/extracellular signal-regulated kinase kinase), an activator of MAP kinase, promotes survival in the absence of NGF (21). However, MAP kinase is not required for the survival of NGF-maintained sympathetic neurons (22, 23) or PC12 cells (24). In contrast, inhibition of PI-3-K blocks the survival-promoting effects of NGF in PC12 cells (24). PI-3-K is a lipid kinase that mediates the mitogenic signal of the platelet-derived growth factor receptor (25). PI-3-K products are involved in regulating mitogenesis, membrane ruffling, glucose uptake, receptor sorting, and receptor down-regulation in response to growth factors (for review, see Ref. 26). In this report, we present evidence that PI-3-K activity is required for survival promotion by K^+ depolarization and insulin-like growth factor I (IGF-I).

EXPERIMENTAL PROCEDURES

Cell Culture Media—The following cell culture media were used: K25+S (Eagle's basal medium (Life Technologies, Inc.) containing 10%

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[1] The abbreviations used are: PCD, programmed cell death; MAP, mitogen-activated protein; PI-3-K, phosphatidylinositol 3-kinase; NGF, nerve growth factor; IGF-I, insulin-like growth factor I; CPT-cAMP, chlorophenylthio-cAMP.

dialyzed fetal bovine serum (Sigma; M_r cutoff = 10,000), 25 mM KCl, 100 units/ml penicillin, and 100 μg/ml streptomycin), K25−S (Eagle's basal medium containing no serum, 25 mM KCl, 100 units/ml penicillin, and 100 μg/ml streptomycin), K5−S (Eagle's basal medium containing no serum, 5 mM KCl, 100 units/ml penicillin, and 100 μg/ml streptomycin).

Neuronal Culture—The cell culture protocol used is extensively detailed by Miller and Johnson (27). In brief, cerebella were dissected from P7 Harlan Sprague Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN), sliced into 1-mm pieces, and incubated at 37 °C for 15 min in 0.30 mg/ml trypsin (Worthington). The tissue was then triturated in K25+S medium with 0.5 mg/ml trypsin inhibitor (Sigma) using a flame-polished Pasteur pipette. The resulting cell suspension was spun at 500 × *g* for 6 min. The pellet was gently triturated in fresh K25+S medium and filtered through a Nitex filter (Tetko Inc., Elmsford, NY). Trypan blue exclusion was used to count the living neurons before plating 2–2.5 × 10^5 cells/cm^2 in either four-well (Nunc, Roskilde, Denmark) or 35-mm (Corning Inc.) dishes. Prior to plating, dishes were coated with 0.1 mg/ml poly-L-lysine (Sigma, P2636). The granule cells were kept at 35 °C in a humidified incubator with 5% CO_2 and 95% air for 7 days. To reduce the number of non-neuronal cells, aphidicolin (3.3 μg/ml; Sigma) was added to the medium 24 h after plating. The cultures were 98–99% pure granule cells; the culture conditions do not support the survival of other neuronal cell types (28, 29), and the non-neuronal contamination was 1–2% (27).

All cultures were treated 7 days after plating. For survival studies, culture dishes were rinsed twice with K5−S medium before adding medium containing 25 mM potassium, LY 294002 (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA), wortmannin (Sigma), PD98059 (New England Biolabs Inc., Beverly, MA), 10 μg/ml cycloheximide (Sigma), 800 μM chlorophenylthio-cAMP (CPT-cAMP; Boehringer Mannheim), or 100 ng/ml IGF-I (a generous gift from Monsanto Co., St. Louis, MO) or a combination of these reagents. Cell viability was quantified from photomicrographs of representative fields of cells labeled with calcein AM (Molecular Probes, Inc., Eugene, OR). Calcein AM is an acetoxymethyl ester fluorescein derivative that is cleaved and trapped inside viable cells that have nonspecific esterase activity (30). The photomicrographs were both taken and scored by a naive observer. Eight photomicrographs at a magnification × 200 were taken for each condition, and the number of calcein AM-positive cells was counted. Cellular nuclei were visualized by fixing cells with 4% paraformaldehyde and staining with 1 μg/ml Hoechst 33258 (Molecular Probes, Inc.).

PI-3-K Assay—Cells were washed three times with K5−S medium, incubated at 35 °C in 5% CO_2 for 3 h, and then stimulated by adding concentrated factors directly to the culture medium. Cells were lysed in PI-3-K lysis buffer as described by Newberry and Pike (31). Approximately 400 μg of lysate protein and 35 μl of anti-phosphotyrosine antibody conjugated to agarose (Calbiochem) were added to the samples and incubated for 2 h at 4 °C. Immune complexes were isolated by centrifugation and washed twice with phosphate-buffered saline (2.7 mM KCl, 140 mM NaCl, 1.5 mM KH_2PO_4, and 10 mM Na_2HPO_4, pH 7.4) plus 1% Triton X-100; twice with 0.5 mM LiCl and 10 mM Tris, pH 7.5; and twice with 10 mM Tris, pH 7.5, 100 mM NaCl, and 1 mM EDTA. PI-3-K activity was assayed in an *in vitro* kinase reaction, and the products were separated by thin layer chromatography (32). Phosphatidylinositol 3-phosphate was identified based on its migration relative to that of a radiolabeled phosphatidylinositol phosphate marker (33). Radioactivity was visualized and quantified with a PhosphorImager (Molecular Dynamics, Inc., Sunnyville, CA).

Phospho-specific MAP Kinase Western Blots—Samples were homogenized in SDS sample buffer and subjected to 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gels were transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Blots were incubated for 1 h at 25 °C in Buffer A (phosphate-buffered saline containing 0.1% Tween 20 and 5.0% (w/v) nonfat dry milk) and then for 16 h at 4 °C with a phospho-specific MAP kinase antibody (New England Biolabs Inc.) diluted 1:1000 in phosphate-buffered saline plus 0.05% Tween 20 and 5% bovine serum albumin. The membranes were then washed in Buffer A (3 × 15 min) and incubated for 1 h in Buffer A containing a 1:1000 dilution of alkaline phosphatase-conjugated anti-rabbit antibody (New England Biolabs Inc.). This was followed by three washes (15 min/wash) in Buffer A; two washes (10 min/wash) in phosphate-buffered saline plus 0.1% Tween 20; and two washes (10 min/wash) in 1 mM $MgCl_2$, 10 mM NaCl, and 10 mM Tris-HCl, pH 9.5. The blot was then incubated for 5 min in CDP-Star (Tropix Inc., Bedford, MA) and exposed to x-ray film.

Equal loading of samples was checked by stripping the blots and reprobing with an antibody that detects total MAP kinase (New England Biolabs Inc.). The membranes were stripped by incubation for 1 h at 25 °C in 1.0% Tween 20, 0.1% SDS, and 0.2 M glycine, pH 2.2. To verify that the blots were completely stripped, they were washed as described above and developed using the CDP-Star reagent. The blots were returned to Buffer A for 1 h and then probed as described above with the total MAP kinase antibody at a 1:1000 dilution.

Determination of Intracellular Calcium Concentration—Cultures were incubated for 45–60 min in K25−S medium with 4 μM fura-2 (Molecular Probes, Inc.) (34) and then washed three times with Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.7 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.6 mM D-glucose, and 5 mM Hepes, pH 7.4) containing 5 mM potassium, 25 mM potassium, or 25 mM potassium plus 30 μM LY 294002. After ~1 h at 35 °C, the cultures were transferred to the heated stage (35 °C) of an inverted fluorescence microscope and illuminated with a 75-watt xenon lamp. Fields containing ~50 neurons were viewed using a 1.3NA ×40 epifluorescent objective (Nikon Inc.). The cultures were alternately illuminated at 340 and 380 nm by switching the excitation filters. The fluorescent signal was detected by an intensified charge-coupled device camera that was connected to a personal computer for image acquisition and analysis (Metafluor, Universal Imaging Corp.). Data represent means ± S.D. for three independent experiments in which ratios were determined for 150–200 cells/condition/experiment.

RESULTS

Depolarization Induces MAP Kinase and PI-3-K Activities—To determine whether depolarizing concentrations of extracellular potassium increase MAP kinase or PI-3-K activity, cerebellar granule cells were switched to K5−S medium for 3 h before the medium was raised to 25 mM by the addition of 2.0 M K^+. Serum-free medium was used in all experiments to isolate the survival-promoting activity of K^+ alone; in granule cells, serum provides survival-promoting activity of unknown origin (27). For PI-3-K activity, neuronal cultures were lysed, immunoprecipitated with a phosphotyrosine antibody, and assayed for PI-3-K activity. Thirty min after treatment, the cultures in 25 mM potassium showed increased PI-3-K activity (Fig. 1*A*). In three independent experiments, PI-3-K activity from depolarized samples was 3.0 ± 0.2-fold above the controls. To verify that the observed signal was attributable to PI-3-K activity, 30 μM LY 294002, a PI-3-K inhibitor, was included in the *in vitro* labeling reaction (Fig. 1*A*, *lane 5*). LY 294002 does not inhibit phosphatidylinositol 4-kinase, which could contribute to the observed signal, nor does it have any effect on protein kinase C, protein kinase A, MAP kinase, S6 kinase, c-Src kinase, or diacylglycerol kinase (35).

Potassium depolarization also caused an activation of the MAP kinase pathway. Samples lysed 15 min after treatment with 25 mM potassium were subjected to Western blot analysis. Probing the blot with a phospho-MAP kinase antibody showed that depolarization led to MAP kinase phosphorylation (Fig. 1*B*, *upper panel*). The blot was stripped and reprobed with an antibody that recognizes both the phosphorylated and non-phosphorylated forms of the ERK1 and ERK2 (extracellular signal-regulated kinase) isoforms of MAP kinase to verify that different lanes contained comparable amounts of sample (Fig. 1*B*, *lower panel*). The finding that K^+ increased both PI-3-K and MAP kinase activities raised the possibility that one or both of these pathways are important for granule cell survival.

MAP Kinase Activation Is Not Required for Potassium-mediated Survival—To determine whether the MAP kinase pathway is important for granule cell survival, cultures were treated with the MEK inhibitor PD98059 (36, 37). Seven-day cultures were switched to K5−S medium for 2.5 h, treated for 30 min with PD98059, and then depolarized by the addition of potassium to 25 mM. To assure that the inhibition was sustained during the time the neurons would become irreversibly committed to die (27), fresh medium and MEK inhibitor were added at 12 and 24 h. Forty-eight h after treatment, neuronal survival was quantified from photomicrographs of calcein AM-

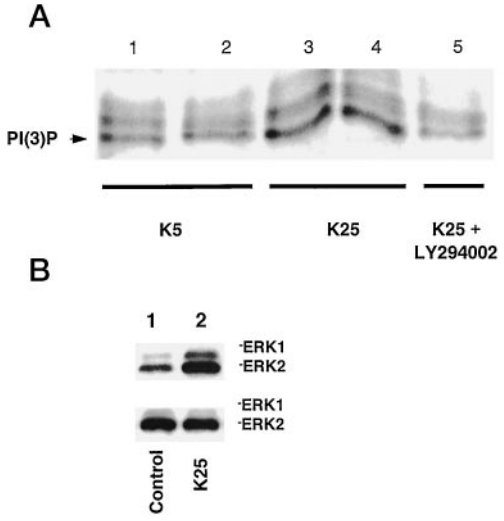

FIG. 1. **Potassium depolarization activates the PI-3-K and MAP kinase pathways.** *A*, cerebellar granule cells were maintained for 7 days and then deprived of K^+ and serum (K5−S medium). After 3 h, the potassium in the K5−S medium was raised to 25 mM by the addition of 20 μl of 2.0 M K^+ (*lanes 3–5*). Water was added to the control cultures (*lanes 1* and *2*). Thirty min after treatment, cultures were lysed, immunoprecipitated with antibodies to phosphotyrosine, and assayed for PI-3-K activity. LY 294002, an inhibitor of PI-3-K activity, was added to the reaction mixture of *lane 5*. *B*, 7-day neuronal cultures were switched to K5−S medium for 2 h before treatment with water (*lane 1*, *Control*) or 25 mM K^+ (*lane 2*, *K25*). Ten min after treatment, samples were lysed, subjected to electrophoresis on a 10% polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane. The membrane was probed with a phospho-MAP kinase antibody (*upper panel*) and then stripped and reprobed with an antibody that recognizes phosphorylated and non-phosphorylated ERK1 and ERK2 isoforms of MAP kinase (*lower panel*). *PI(3)P*, phosphatidylinositol 3-phosphate.

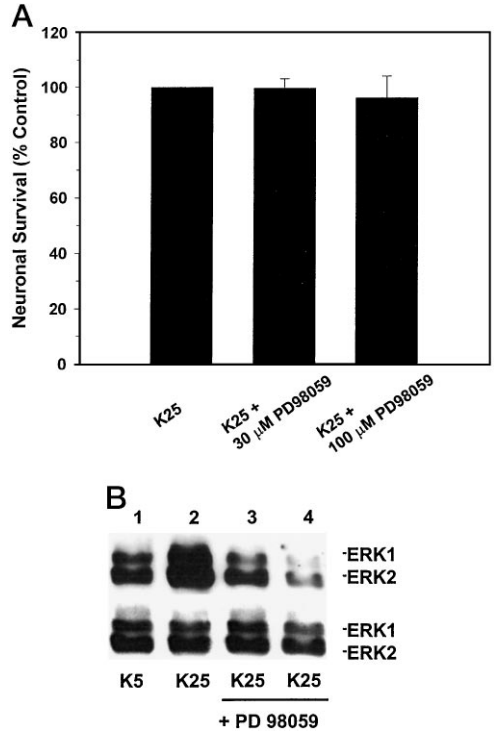

FIG. 2. **Inhibition of MEK1 does not block K^+-mediated neuronal survival.** *A*, 7-day cerebellar granule cell cultures were switched to K25−S medium, K25−S medium plus 30 μM PD98059, or K25−S medium plus 100 μM PD98059. Neuronal survival was assayed after 48 h by counting calcein AM-stained neurons in photomicrographs of cultures. *B*, 7-day cultures were deprived of K^+ and serum for 2.5 h and then treated with or without the MEK inhibitor PD98059 for 30 min before the potassium was raised to 25 mM. *Lane 1*, control; *lane 2*, 25 mM K^+; *lane 3*, 30 μM PD98059 and then 25 mM K^+; *lane 4*, 100 μM PD98059 and then 25 mM K^+. *Upper panel*, probed with phospho-MAP kinase antibody; *lower panel*, stripped and reprobed with an antibody that recognizes phosphorylated and non-phosphorylated ERK1 and ERK2 isoforms of MAP kinase.

stained cultures. Inhibiting the activation of MAP kinase with either 30 or 100 μM PD98059 had no apparent effect on the ability of potassium to promote survival (Fig. 2*A*). Phospho-MAP kinase Western blots revealed that these doses of PD98059 were sufficient to inhibit MAP kinase activation (Fig. 2*B*), and this inhibition was sustained even 24 h after treatment.[2] Thus, the events downstream of MAP kinase activation were not required for potassium-mediated survival of granule neurons.

Inhibition of PI-3-K Kills Depolarization-maintained Neurons—Since LY 294002 can block PI-3-K in intact cells (24, 35), we also used this compound to determine whether PI-3-K activity is involved in the survival-promoting effect of K^+ depolarization. Cultures were switched to K25−S medium either with or without LY 294002, and cell survival was assayed 48 h later by counting the number of cells in photomicrographs of calcein AM-stained cultures. LY 294002 induced a dose-dependent cell death of granule cells with an EC_{50} of ~20 μM (Fig. 3*A*, *closed squares*). This dose-response curve is consistent with that of LY 294002 for inhibition of PI-3-K (35).

When granule cells are switched to K5−S medium, the neurons die by apoptosis (17). One property of this PCD is its inhibition by cycloheximide. LY 294002-induced cell death was also blocked by cycloheximide (Fig. 3*A*, *open squares*). The addition of cycloheximide concurrent with the PI-3-K inhibitor prevented the dose-dependent killing, indicating that inhibition of PI-3-K triggered PCD.

Similar results were obtained with wortmannin, a fungal metabolite that also blocks PI-3-K. Wortmannin caused a dose-dependent killing (Fig. 3*B*, *closed squares*) that was blocked by cycloheximide (*open squares*). Because wortmannin is a labile compound (38), the culture medium was changed every 6 h during the first 24 h of treatment. Thus, two inhibitors of PI-3-K that act via distinct mechanisms block the survival-promoting ability of potassium depolarization. If this killing is truly caused by a disruption in a specific survival pathway, then the death induced by the PI-3-K inhibitors should share features of that induced by switching to K5−S medium. Because of the lability of wortmannin, these experiments were conducted with LY 294002.

Cell Death Caused by LY 294002 Is Indistinguishable from That Caused by K^+ Removal—Switching to K5−S medium causes the programmed cell death of 60% of granule cells in 48 h (17, 27). To determine whether the cell death induced by PI-3-K inhibition had properties similar to PCD in K5−S medium, we used 30 μM LY 294002, a dose that caused a comparable amount of cell loss. We then compared the cell death caused by LY 294002 with that caused by K5−S medium based on several different criteria including dependence on macromolecular synthesis, morphology of the nuclei of dying cells, and time course of cell loss.

Similar to PCD induced by K5−S medium, the protein synthesis inhibitor cycloheximide (10 μg/ml) blocked the cell death induced by LY 294002 (Fig. 3*A*, *open squares*). Fig. 4 shows the phase-contrast (*panels a–d*) and the corresponding calcein AM-stained (*panels e–h*) images after 48 h and the bisbenzimide-stained nuclei (*panels i–l*) after 18 h of granule cells maintained in K25−S medium (*panels a*, *e*, and *i*), in K25−S medium plus 30 μM LY 294002 (*panels b*, *f*, and *j*), in K5−S medium (*panels*

[2] T. M. Miller, D. J. Creedon, and E. M. Johnson, Jr., unpublished observations.

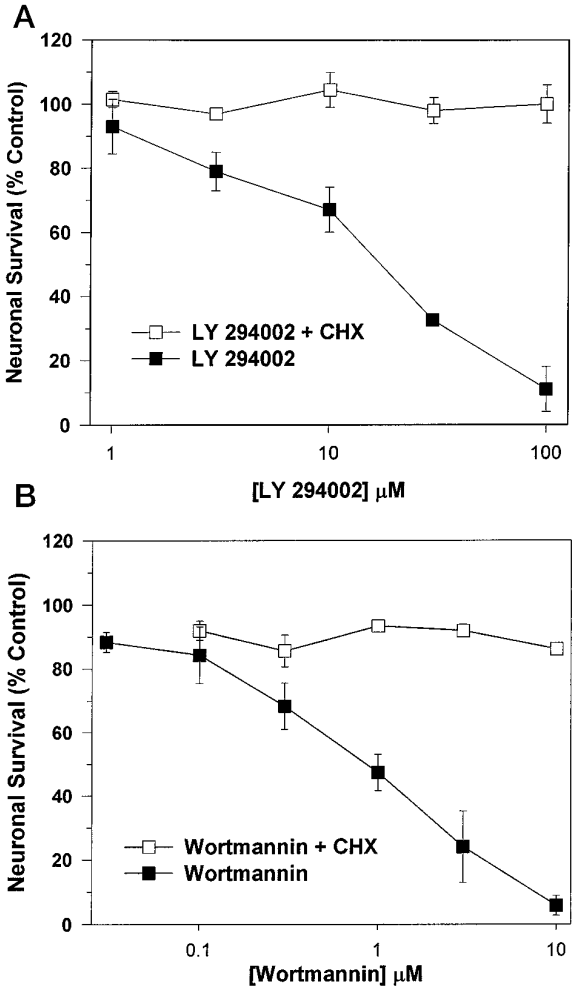

FIG. 3. **LY 294002 and wortmannin cause a dose-dependent cell death that is blocked by cycloheximide.** Seven-day neuronal cultures were switched to K25−S medium with increasing concentrations of LY 294002 (*A*) or wortmannin (*B*) in the absence (*closed squares*) or presence (*open squares*) of 10 μg/ml cycloheximide (*CHX*). Neuronal survival was assayed after 48 h by counting calcein AM-stained neurons in photomicrographs of cultures. Results represent mean ± range from two independent experiments.

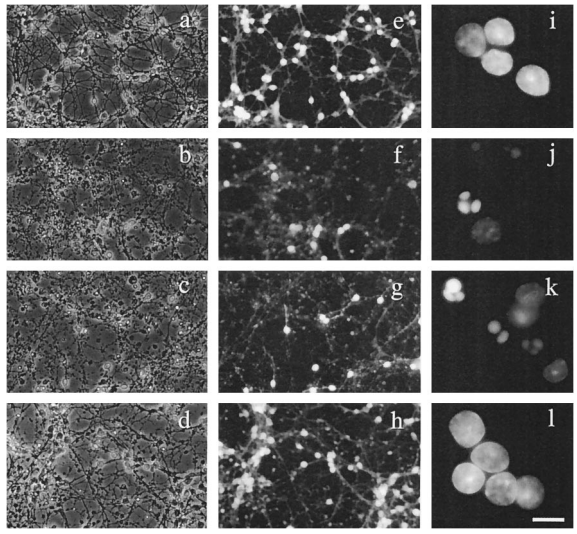

FIG. 4. **Morphological properties of LY 294002-induced cell death are indistinguishable from those of K5−S medium-induced cell death.** Seven-day neuronal cultures were switched to K25−S medium (*panels a*, *e*, and *i*), K25−S medium plus 30 μM LY 294002 (*panels b*, *f*, and *j*), K5−S medium (*panels c*, *g*, and *k*), or K25−S medium plus 30 μM LY 294002 and 10 μg/ml cycloheximide (*panels d*, *h*, and *l*). *Panels a–d* are phase-contrast images, and *panels e–h* are the corresponding photomicrographs of calcein AM staining taken after 48 h. *Panels i–l* are photomicrographs of nuclei stained with bisbenzimide after 18 h. The *scale bar* equals 25 μm in *panels a–h* and 5 μm in *panels i–l*.

c, *g*, and *k*), or in K25−S medium plus 30 μM LY 294002 and 10 μg/ml cycloheximide (*panels d*, *h*, and *l*). LY 294002 blocked the survival-promoting effect of 25 mM potassium (compare *panels a* and *e* and *panels b* and *f*) and led to a cell death that resembled the programmed cell death induced by K5−S medium (*panels c* and *g*). Cycloheximide prevented the LY 294002-induced cell death (*panels d* and *h*) as it prevents PCD in K5−S medium (17). The nuclear morphology of granule cells treated with LY 294002 also resembled the nuclei of neurons undergoing K5−S medium-induced PCD. In contrast to the round and homogeneously stained nuclei of cells maintained in 25 mM K^+ (*panel i*), the nuclei of neurons in K25−S medium plus LY 294002 (*panel j*) and those in K5−S medium (*panel k*) were condensed and fragmented, features typical of apoptotic nuclei. Again, however, cycloheximide was able to block the effects of PI-3-K inhibition. The nuclei of granule cells in K25−S medium plus LY 294002 and cycloheximide (*panel l*) were indistinguishable from controls (*panel i*).

Further evidence that inhibition of PI-3-K is similar to removal of K^+ was found in the time course of death. After 6, 12, 24, or 48 h, the number of remaining cells was assessed by counting photomicrographs of calcein AM-stained cultures. The resulting time course of cell loss was identical in cultures switched to K5−S medium or to K25−S medium plus LY 294002 (Fig. 5). Together, these results suggest that inhibiting PI-3-K mimicked the programmed cell death that accompanies a switch to low K^+.

LY 294002 Does Not Interfere with the Depolarization-induced Increase in Intracellular Calcium—Since the survival-promoting effect of potassium depolarization is critically dependent on an increase in intracellular calcium (15), we tested whether LY 294002 had any effect on the sustained rise in intracellular calcium in depolarized cells. Intracellular calcium was measured by determining 340/380 nm ratios for the calcium-sensitive dye fura-2. An increase in the 340/380 nm ratio is indicative of an increase in intracellular free calcium. K^+ depolarization of granule cells caused an ~2-fold increase in the 340/380 nm ratio that was not blocked by 30 μM LY 294002 (Fig. 6). Thus, blocking calcium channels or the sustained rise in intracellular calcium was not the mechanism by which LY 294002 blocked the survival-promoting activity of depolarizing concentrations of K^+.

Neurons Saved by IGF-I or cAMP Differ in PI-3-K Activation and Their Susceptibility to Cell Death Caused by LY 294002—In addition to potassium depolarization, cerebellar granule cells can be maintained *in vitro* in low K^+ medium supplemented with either IGF-I or CPT-cAMP (17). We found that, similar to K^+ (Fig. 1), IGF-I (100 ng/ml) activated PI-3-K, whereas CPT-cAMP (800 μM) did not (Fig. 7*A*). In accordance with their ability to stimulate PI-3-K, LY 294002 blocked the survival-promoting effect of IGF-I and K^+, but not that of CPT-cAMP (Fig. 7*B*). At 48 h, K5−S medium plus IGF-I or plus CPT-cAMP promoted granule cell survival comparable to K25−S medium. As with K25−S medium, LY 294002 reversed the saving effects of IGF-I. In fact, the dose-response curve for LY 294002 killing of IGF-maintained cells (Fig. 7*C*) mirrored the effect of LY 294002 on potassium-saved neurons (Fig. 3*A*). In contrast, cells maintained in CPT-cAMP survived despite the presence of the PI-3-K inhibitor.

Since IGF-I stimulates a tyrosine kinase receptor, the MAP

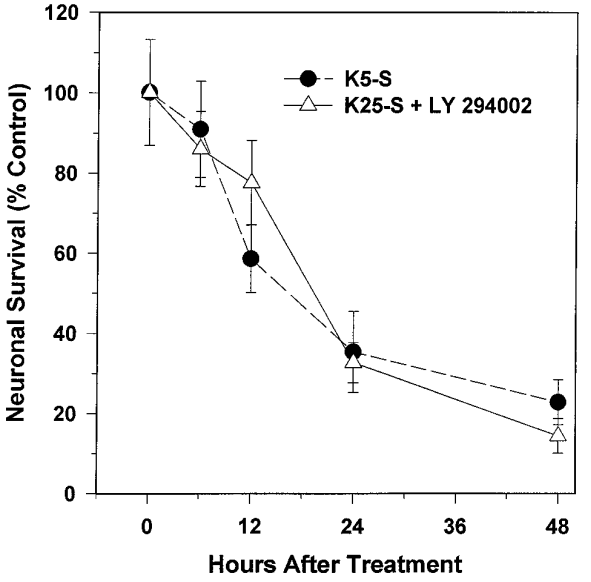

FIG. 5. **Time courses of LY 294002-induced and K5−S medium-induced cell death are indistinguishable.** Seven-day neuronal cultures were switched to K5−S medium (*closed circles*) or K25−S medium plus 30 μM LY 294002 (*open triangles*). Neuronal survival was assayed after 6, 12, 24, or 48 h from photomicrographs of calcein AM-stained cultures and compared with control neurons maintained in K25−S medium. Results represent mean ± range from two independent experiments.

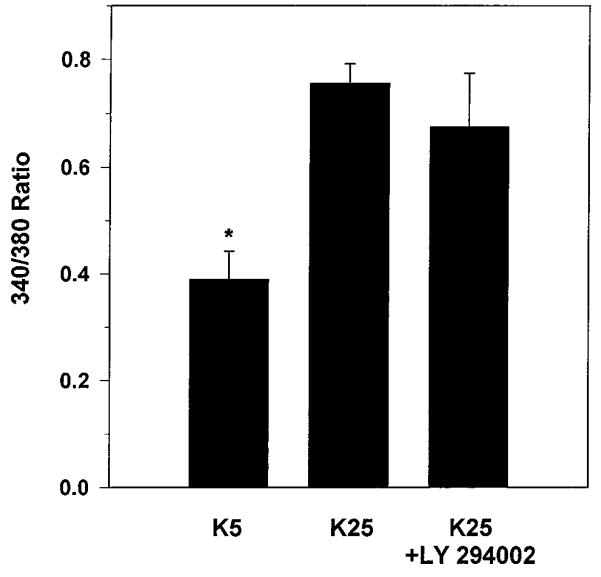

FIG. 6. **LY 294002 does not block the sustained rise in intracellular calcium that accompanies depolarization.** Seven-day neuronal cultures were loaded with 4 μM fura-2 for 45 min. Cultures were then washed and switched to K5−S medium, K25−S medium, or K25−S medium plus 30 μM LY 294002. After ~60 min, 340/380 nm ratios were determined. Results represent mean ± S.D. of three independent experiments. *, $p < 0.05$ when an analysis of variance was used to compare results with K25−S medium.

kinase pathway is another candidate for survival-promoting activity. Like the potassium-maintained cells, however, MEK inhibition had no discernible effect on IGF-I saving (Fig. 7*C*, *closed circles*) or on CPT-cAMP-maintained neurons.[2] The fact that IGF-I survival was blocked by LY 294002 implies that both K^+ and IGF-I promote survival by signaling through PI-3-K, whereas CPT-cAMP appears to act downstream of PI-3-K or through a non-PI-3-K-dependent pathway. The failure of LY 294002 to block CPT-cAMP-mediated survival also provides additional evidence that LY 294002 is not generally toxic to

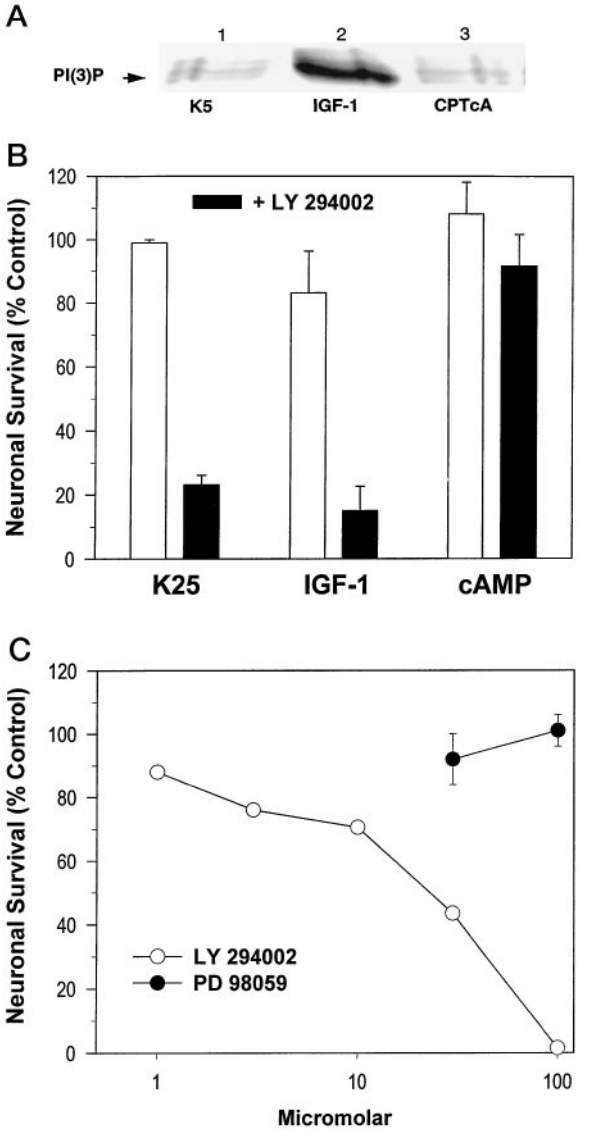

FIG. 7. **IGF-I and cAMP differ in PI-3-K activation and their ability to promote survival in the presence of LY 294002.** *A*, 7-day neuronal cultures were switched to K5−S medium. After 3 h, water (*lane 1*), 100 ng/ml IGF-I (*lane 2*), or 800 μM CPT-cAMP (*CPTcA*; *lane 3*) was added to the cultures. Thirty min after treatment, cultures were lysed, immunoprecipitated with antibodies to phosphotyrosine, and assayed for PI-3-K activity. *B*, 7-day neuronal cultures were switched to K25−S medium, K5−S medium plus 100 ng/ml IGF-I, or K5−S medium plus 800 μM CPT-cAMP, either in the absence (*open bars*) or presence (*closed bars*) of 30 μM LY 294002. Neuronal survival was determined from calcein AM-stained cultures 48 h after treatment. *C*, 7-day neuronal cultures were switched to K5−S medium plus 100 ng/ml IGF-I with increasing concentrations of LY 294002 (*open circles*) or PD98059 (*closed circles*). Neuronal survival was assayed after 48 h by calcein AM staining. Results represent mean ± range from two independent experiments. *PI(3)P*, phosphatidylinositol 3-phosphate.

cerebellar granule cells; these results support a role for PI-3-K in neuronal survival.

DISCUSSION

In this study, we provide evidence for a link between K^+ depolarization and a signaling pathway that promotes the survival of neurons. Depolarizing concentrations of extracellular potassium increased PI-3-K activity; that this activity is critical for survival is indicated by the ability of inhibitors of PI-3-K to block depolarization-mediated survival. The cell death resulting from inhibition of PI-3-K was indistinguishable from the programmed cell death triggered by lowering the K^+ con-

centration. In both cases, cell death was dependent on macromolecular synthesis and accompanied by morphologically apoptotic nuclei. In addition, the time course of cell loss was identical, and the cell death was prevented by cAMP. These data strongly suggest that PI-3-K inhibition resulted in programmed cell death by specifically blocking the survival-promoting activity of K^+ depolarization.

The survival-promoting activity of IGF-I was also blocked by inhibition of PI-3-K. K^+ depolarization and growth factors may therefore converge on PI-3-K to signal survival promotion. PI-3-K activity increases in response to several growth factors including platelet-derived growth factor, insulin, colony-stimulating factor 1, nerve growth factor, hepatocyte growth factor, stem cell growth factor, and epidermal growth factor (26). As previously reported in cell lines (39, 40), we found that IGF-I increased PI-3-K activity in cerebellar granule neurons. Although both IGF-I and 25 mM potassium increased PI-3-K activity and inhibiting PI-3-K blocked their ability to promote survival, we did not directly test whether the rise in PI-3-K activity *per se* was critical for survival. Basal activity could augment other actions of these agents that result in survival. In the absence of 25 mM potassium or IGF-I, however, the basal PI-3-K activity was not sufficient to maintain the neurons.

For growth factors, activation of PI-3-K is dependent on the protein-tyrosine kinase activity of the growth factor receptor. Autophosphorylation of specific tyrosine residues on the receptor recruits the Src homology 2 domain (SH2) of the p85 regulatory subunit of PI-3-K. This leads to relocalization of PI-3-K to the membrane and activation of the catalytic p110 subunit. In contrast to other known growth factors, phosphorylation of the insulin or IGF-I receptor leads to phosphorylation of insulin receptor substrate 1, which serves as an SH2-docking protein for p85 (39, 40–42).

One mechanism by which potassium depolarization may increase PI-3-K activity is by causing phosphorylation events similar to those caused by growth factor receptors. Some evidence supports this hypothesis. For example, PYK2, a novel protein-tyrosine kinase, is rapidly phosphorylated in response to stimuli that increase intracellular calcium (43). The p85 subunit of PI-3-K may associate with this calcium-responsive protein-tyrosine kinase. Alternatively, PYK2 may activate PI-3-K by first activating Ras, which can be an upstream activator of PI-3-K (44). Potassium depolarization of PC12 cells results in phosphorylation of the adapter protein Shc and leads to the association of Shc with Grb2, causing Ras and MAP kinase activation (45–47) and phosphorylation of the epidermal growth factor receptor (47). At this point, which of these phosphorylation events occurs in depolarized granule cells is unclear.

In cultures of cortical neurons, depolarization promotes survival by stimulating the release of the neurotrophin brain-derived neurotrophic factor that serves as an autocrine or paracrine trophic agent (48). This contrasts with sympathetic neurons, however, in which potassium depolarization fails to induce the phosphorylation of growth factor receptors (10). In granule cell cultures, antibodies to IGF-I, neurotrophin-3, NGF, or brain-derived neurotrophic factor do not affect granule cell survival in depolarizing medium,[2] suggesting that granule cells behave similarly to sympathetic neurons in this regard. Another mechanism whereby calcium may influence survival is by increasing calcium/calmodulin kinase activity. Inhibitors of calmodulin- or Ca^{2+}/calmodulin-dependent protein kinase II activity block K^+-mediated survival (15, 49). However, these compounds may affect survival by blocking the sustained rise in intracellular calcium (10).

We found that LY 294002 did not interfere with increases in intracellular calcium after 1 h (Fig. 6), a time when inhibitors of calcium channels, such as nifedipine, will block calcium channels completely (10). It is possible that LY 294002 affected calcium at later time points, but measurement of calcium at later time points would be difficult to interpret because the cells begin to degenerate within 6 h, and changes in metabolic parameters such as glucose uptake and protein synthesis occur within 2 h of the induction of apoptosis (27). In addition to our direct measurement of calcium at 1 h, two other pieces of evidence argue against LY 294002 affecting calcium. First, since the time course of death triggered by K5−S medium or by K25−S medium plus LY 294002 overlaps (Fig. 5), an LY 294002 effect on calcium would have to occur rapidly. If LY 294002 caused a delayed decrease in intracellular calcium that initiated PCD, we would expect to see a corresponding delay in the cell death time course, but this was not the case (Fig. 5). Second, LY 294002 blocked the survival-promoting effect of IGF-I, which does not elevate intracellular calcium (11).

An indication that Ras is activated by depolarization is that depolarization activates the MAP kinase pathway (Fig. 6) (45–47, 50). In PC12 cells, overexpression of a constitutively active form of MEK1, an activator of MAP kinase, promotes survival in the absence of NGF (21). However, MAP kinase is not required for the survival of NGF-maintained sympathetic neurons (22, 23) or NGF-maintained PC12 cells (24). Consistent with this, our data demonstrate that MAP kinase was not required for survival because the MEK inhibitor, which blocks activation of MAP kinase, did not affect survival. The fact that PI-3-K inhibitors do not prevent the activation of MAP kinase[2] yet lead to PCD (Fig. 3) indicates that MAP kinase activity was also not sufficient to promote granule neuron survival.

Several possible downstream targets of PI-3-K may be involved in neuronal survival. Protein kinase C isozymes (51–54), Rac (55, 56), $p70^{S6K}$ (57–59), and AKT/protein kinase B serine/threonine kinase (60, 61) are all effectors of PI-3-K. PI-3-K also affects glucose transporters (62–64) and, through this pathway, may be critical for maintaining the metabolic functions of cells. We have found that glucose uptake, protein synthesis, and RNA synthesis decrease dramatically as granule cells (27) and sympathetic neurons (65) undergo PCD and have suggested that this dramatic decrease in metabolic parameters is part of PCD in neurons. Inhibiting PI-3-K may specifically decrease metabolic functions maintained by either K^+ depolarization or growth factors and thus trigger PCD.

Although peripheral neurons are particularly dependent on growth factors for survival, neurons from the central nervous system may depend on a combination of trophic factors and electrical activity (66). Our results highlight PI-3-K as an important intracellular mediator of survival promotion by either activity or trophic factors. PI-3-K is one of the pathways upon which neurons critically depend for survival *in vitro* and, perhaps, *in vivo*.

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