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Calcium-Dependent Prevention of Neuronal Apoptosis by Lithium Ion: Essential Role of Phosphoinositide 3-Kinase and Phospholipase Cγ

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ABSTRACT

We examined the possibility that the neuroprotective effects of Li^+ would depend upon the patterns of neuronal death, apoptosis versus necrosis, and whether Ca^{2+} as well as phosphoinositide 3-kinase (PI3-K) would mediate the neuroprotective effect of Li^+. Cortical neurons treated with Li^+ showed marked increase in $[Ca^{2+}]_i$ within 2 min. Addition of BAPTA-acetoxymethyl ester, a selective Ca^{2+} chelator, abrogated the antiapoptotic effect of Li^+. PI3-K was activated rapidly within 1 min after exposure to Li^+, which mediated Ca^{2+}-dependent neuroprotective effects of Li^+. Activated PI3-K seemed to increase $[Ca^{2+}]_i$ via the phospholipase Cγ (PLCγ) pathway. Antiapoptosis action of Li^+ was prevented in the presence of U-73122, a selective phospholipase C inhibitor, and was not observed in PLCγ1-null fibroblasts. In contrast to antiapoptosis action, administration of Li^+ did not prevent neuronal cell necrosis by excitotoxicity or free radicals. Li^+ selectively prevents apoptosis by increasing $[Ca^{2+}]_i$ through activation of PI3-K and PLCγ pathways.

Lithium ion (Li^+), the lightest monovalent cation of the alkali metals, acts as a mood-stabilizing agent and is widely used for the acute and prophylactic treatment of bipolar disorder and recurrent depression (Goodwin and Jamison, 1990). Although the precise mechanisms underlying the mood-stabilizing action of Li^+ remain to be elucidated, Li^+ seems to exert its action by modulating neurotransmission, inhibiting G protein-mediated inositol 1,4,5-triphosphate (IP_3) formation, and reducing cAMP production (Manji et al., 1995). Recently, new pharmacological effects of Li^+ have appeared, showing that Li^+ can influence neuronal survivorship. Administration of Li^+ attenuates apoptosis after exposure to low potassium, ceramide, or staurosporine, although it can injure immature cerebellar neurons in culture with staurosporine (D'Mello et al., 1994; Centeno et al., 1998; Bijur et al., 2000). Long-term treatment with Li^+ prevents *N*-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity (Nonaka et al., 1998a). Li^+ also attenuates neuronal injury after deprivation of oxygen and glucose or striatal injections of quinolinic acid (Cimarosti et al., 2001; Wei et al., 2001).

Several lines of evidence support the idea that apoptosis and necrosis reveal unique morphology of cytoplasmic and nuclear organelles during degenerative process and propagate through mutually exclusive pathways (Kerr et al., 1972; Choi, 1996). Blocking excitotoxic necrosis with glutamate antagonists unveils caspase-mediated neuronal apoptosis after prolonged deprivation of oxygen and glucose (Gwag et al., 1995; Gottron et al., 1997). Neurotrophins or gangliosides attenuate apoptosis in neuronal cells but markedly potentiate neuronal cell necrosis induced by NMDA or reactive oxygen species (Koh et al., 1995a; Ryu et al., 1999a). Thus, the beneficial effects of neuroprotectants seem to be limited to the patterns (e.g., apoptosis and necrosis) of neuronal death. The present study was performed to examine the possibility that the neuroprotective effects of Li^+ would depend upon types of neuronal cell death, turning to well char-

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ABBREVIATIONS: IP_3, inositol 1,4,5-trisphosphate; NMDA, *N*-methyl-D-aspartate; MK-801, dizocilpine maleate; PI3-K, phosphoinositide 3-kinase; PLCγ, phospholipase Cγ; BAPTA, 1,2-bis(2-Aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropioinc acid; F-127, [poly(ethylene oxide)$_{100}$–poly(propylene oxide)$_{65}$–poly(ethylene oxide)$_{100}$]; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one;U-73122, 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione;U-73343, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5- pyrrolidine-dione; DIV, days in vitro; LDH, lactate dehydrogenase; AM, acetoxymethyl ester; PP, protein phosphatase.

acterized models of apoptosis and necrosis in cortical cell cultures. In addition, we set out experiments to delineate whether the neuroprotective actions of Li^+ are mediated through modulation of PI3-K and Ca^{2+}, the two upstream signals indispensable for neuronal survivorship.

Materials and Methods

Materials. Lithium chloride, ferrous chloride, cycloheximide, and cytosine arabinoside were purchased from Sigma (St. Louis, MO). NMDA, MK-801, 6-cyano-7-nitroquinoxalene-2,3-dione, and AMPA were obtained from RBI/Sigma (Natick, MA). Trolox was obtained from Aldrich (Milwaukee, WI). BAPTA-acetoxymethyl ester (AM), pluronic F-127, and fluo-3 AM were purchased from Molecular Probes (Eugene, OR). Caliculin A, cyclosporin A, wortmannin, LY294002, U-73122 and U-73343 were obtained from Calbiochem (La Jolla, CA). Phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY). IP_3 assay kit was purchased from Amersham Biosciences (Piscataway, NJ).

Cell Culture. Cortical cells were prepared from fetal ICR mice (embryonic day 15) and mechanically triturated. Dissociated cells were plated on 24-well plates (five hemispheres/plate, approximately 10^5 cells/well) in a plating medium consisting of Eagle's minimal essential media supplemented with 5% horse serum, 5% fetal bovine serum, 2 mM glutamine, and 21 mM glucose. Proliferation of non-neuronal cells was halted by adding cytosine arabinoside (final concentration, 10 μM) at 7 to 9 days in vitro (DIV 7–9) when astrocytes became confluent. Cultures were then fed twice a week with plating medium lacking fetal serum. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Assessment of Cell Death. In mixed cortical cell cultures of neurons and glia, neuronal cell apoptosis was induced at ages of DIV 10–12, for mature cortical neurons (>DIV 14) are resistant to apoptosis. Excitotoxicity or oxidative stress was performed in cortical cell cultures (DIV 10–14). Neuronal death was analyzed 24 h later by measuring LDH release, and the percentage of neuronal death was normalized to the mean LDH value released after complete death of neurons after 24 h-exposure to 500 μM NMDA (100%) or a sham control (0%) as described previously (Koh and Choi, 1987). Because fibroblast cells are insensitive to NMDA, the percentage of cell death was normalized to the mean LDH value after complete lysis of fibroblasts with 0.2% Triton X-100 (100%) or a sham control (0%).

Annexin V Binding and Propidium Iodide Staining Assay. Apoptotic neuronal cell death was visualized by using Annexin-V-FLUOS staining kit from Roche (Manheim, Germany). Cortical cell cultures grown in glass-bottomed dishes were incubated with Annexin V-FLUOS and 5 μg/ml propidium iodide for 10 min at room temperature in the dark. Cells were then observed using a confocal scanning laser microscopy (FLUOVIEW FV300; Olympus, Tokyo, Japan). The laser scanning microscope was used in the dual parameter setup, according to the manufacturer's specification, using dual wavelength excitation at 488 nm and 568 nm.

Calcium Imaging. Measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$) was carried out using the Ca^{2+} sensitive indicator fluo-3 under a fluorescence microphotometry. Cortical cell cultures (DIV 11) grown on a glass-bottomed dish were loaded with 5 μM fluo-3 AM plus 2% Pluronic F-127 for 30 min at room temperature. Cells were washed three times with a salt solution containing 120 mM NaCl, 5 mM KCl, 2.3 mM $CaCl_2$, 15 mM glucose, 20 mM HEPES, and 10 mM NaOH, pH 7.4. The fluo-3 fluorescent signals (excitation at 490 nm, emission at 510 nm) were acquired with a Nikon Diaphot inverted microscope and charge-coupled device. Fluo-3 fluorescence images were collected at 10-s intervals and analyzed using a Quanticell 700 system (Applied Imaging, Sunderland, UK). Changes in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) were estimated as $\Delta F/F0$, where ΔF was defined as a Li^+-induced fluorescent intensity after subtracting the basal fluo-3 intensity, and $F0$ was derived from the averaged intensity of the first 10 to 20 frames minus the background in the cell-free region (Kao et al., 1989).

$^{45}Ca^{2+}$ Uptake. Cultures were washed with buffer (120 mM NaCl, 5 mM KCl, 2.3 mM $CaCl_2$, 15 mM Glucose, 20 mM HEPES, and 10 mM NaOH, pH 7.4), added with $^{45}Ca^{2+}$ solutions (1 μCi/ml) containing 5 mM Li^+ or 100 μM NMDA, and incubated for indicated points of time. Cultures were washed with the same buffer, lysed with 0.2% SDS, and subjected to measurement of $^{45}Ca^{2+}$ radioactivity.

PI3-K Assay. Cultures were lysed in an ice-cold lysis buffer containing 137 mM NaCl, 20 mM Tris, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol (v/v), 1% Nonidet P40, 1 mM phenylmethylsulfonyl fluoride, and 200 μM vanadate. The lysates were centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected, and protein concentration was determined using the DC protein assay kit (Bio-Rad, Hercules, CA). A protein sample of ~600 μg was reacted with 5 μg of a mouse monoclonal antibody specific for the p110 subunits of PI3-K (Santa Cruz, CA) for overnight at 4°C. The immunoprecipitates were bound to the protein A-Sepharose beads (5 mg/μg), which was then washed with buffer I (137 mM NaCl, 15.7 mM NaH_2PO_4, 1.47 mM KH_2PO_4, 2.68 mM KCl, 1% Nonidet P40, and 200 μM vanadate), buffer II (100 mM Tris, pH 7.5, 500 mM NaCl, and 200 μM vanadate), and buffer III (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA pH 7.5, and 200 μM vanadate). To analyze the activity of PI3-K, each immunoprecipitated sample was incubated for 10 min at 25°C in a reaction mixture containing 10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA pH 7.5, 20 mM $MgCl_2$, 0.5 mg/ml sonicated propidium iodide, [^{32}P]ATP, and unlabeled ATP. This kinase reaction was stopped by adding 20 μl of HCl (8 M) and 160 μl of methanol/chloroform (1:1). The lower organic phase was recovered and spotted on 1% oxalate-coated silica gel thin-layer chromatography plate. The plate was developed in chloroform/methanol/water/ammonium hydroxide (120:94:23.2:4) for 30 to 60 min. The plate was exposed on X-ray film with an intensifying screen.

Western Blot Assay. Cells were lysed in a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 100 μg/ml leupeptin. Lysates were centrifuged at 13,000g for 10 min. Supernatants were collected, subjected to electrophoresis on a 12% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. The blot was incubated in 5% nonfat dry milk for 30 min, reacted with primary antibodies for overnight at 4°C, and then incubated with a biotinylated anti-rabbit secondary antibody for 4 h. Signals were detected using the Vectastain avidin:biotinylated enzyme complex kit (Vector Labs, Burlingame, CA) and luminol as an enhanced chemiluminescence substrate (Amersham Biosciences, Buckinghamshire, England), and then analyzed using an image analyzer LAS1000 (Fuji, Tokyo, Japan).

IP_3 Assay. After lithium treatment, aliquots of cell suspension were transferred to 0.2 volumes of ice-cold perchloric acid [20% (w/v)], mixed thoroughly, left on ice for 20 min and centrifuged at 2000g for 15 min at 4°C. The supernatant was transferred into a plastic tube, neutralized to pH 7.5, and centrifuged at 2000g for 15 min at 4°C. The soluble fraction containing IP_3 was incubated on ice with ~0.75 μCi of [^{3}H]IP_3 and D-*myo*-inositol 1,4,5-triphosphate bovine adrenal binding protein for 15 min, and centrifuged at 2000g for 10 min at 4°C. The pellet was resuspended and its radioactivity was measured in β-scintillation counter. Nonspecific binding of [^{3}H]IP_3 was determined in excess of nonradioactive IP_3 (Amersham Biosciences).

PLCγ-Null Fibroblast Culture. Mouse embryo fibroblasts were maintained at 37°C in a humidified atmosphere (5% CO_2 in air) in Dulbecco's minimal essential medium containing 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin.

Results

Li^+ Selectively Attenuated Neuronal Apoptosis. We first examined whether Li^+ would prevent neuronal cell apoptosis in cortical cell cultures. As reported previously (McDonald et al., 1996; Ko et al., 2000), administration of cyclosporin A, a selective inhibitor of phosphatase (PP) 2B, or calyculin A, a selective inhibitor of PP1 and PP2A, produced shrinkage and apoptotic death of cultured cortical neurons over the next 24 h. This death was prevented by inclusion of cycloheximide, a protein synthesis inhibitor that was known to block apoptotic death of postmitotic cells (Fig. 1a). Concurrent treatment with 3 to 30 mM Li^+ showed dose-dependent protection against calyculin A- or cyclosporin A-induced neuronal apoptosis. At these doses, Li^+ almost completely blocked apoptosis of cortical neurons deprived of serum (H. J. Kang, B. J. Gwag, unpublished observations), suggesting that Li^+ protects neurons from apoptotic injuries. This range of concentrations of Li^+ has been used to prevent inositol monophosphate phosphatase, glycogen synthase kinase-3, glutamate uptake, and cell death (Hallcher and Sherman, 1980; Hong et al., 1997; Dixon and Hokin, 1998; Nonaka et al., 1998b; Hoeflich et al., 2000).

We next tested whether Li^+ would influence the excitotoxicity that causes necrotic degeneration of neuronal cells made evident by marked swelling of cell body and mitochondria (Gwag et al., 1997). Concurrent treatment with Li^+ prevented neither early swelling nor late necrosis of cortical neurons briefly exposed to 20 μM NMDA or 30 μM AMPA (Fig. 1b). Interestingly, Li^+ significantly enhanced NMDA-induced neuronal death. Thus, whereas long-term pretreatment with Li^+ seems to prevent excitotoxicity by reducing NMDA receptor-mediated Ca^{2+} influx (Nonaka et al., 1998a), concurrent and short-term treatment with Li^+ is not beneficial against NMDA neurotoxicity. Cortical neurons underwent necrotic degeneration after exposure to Fe^{2+}, a hydroxyl radical-producing agent through Fenton chemistry, as previously reported (Ryu et al., 1999b). Fe^{2+}-induced neuronal cell necrosis was not prevented by inclusion of Li^+. Taken together, the neuroprotective effects of Li^+ seem to be selective against apoptosis without beneficial effects against pro-necrotic insults such as excitotoxicity or oxidative stress.

Increase in $[Ca^{2+}]_i$ Mediates the Antiapoptotic Action of Li^+. We next studied upstream mechanisms underlying the antiapoptosis action of Li^+. In light of ideas that neurons can be rescued from apoptosis or programmed cell death with supplement of appropriate Ca^{2+} as well as neurotrophic factors (Franklin and Johnson, 1994), we examined whether Ca^{2+} would mediate the antiapoptosis action of Li^+. Cortical neurons treated with Li^+ revealed an immediate increase in $[Ca^{2+}]_i$. This increase was maximally observed within 2 min after exposure to Li^+ (Fig. 2, a and b). Thereafter, Li^+-induced increase in $[Ca^{2+}]_i$ rapidly declined but remained up to 2.5-fold higher than basal level until analyzed over 8 min. Additional experiments were performed to determine whether Li^+-induced increase in $[Ca^{2+}]_i$ was attributed to influx of extracellular Ca^{2+}. Whereas administration of NMDA resulted in marked influx of $^{45}Ca^{2+}$, treatment with Li^+ did not significantly change influx of $^{45}Ca^{2+}$ over 30 min (Fig. 2c). Thus, Li^+ seems to increase $[Ca^{2+}]_i$ by modulating transport of intracellular Ca^{2+}. Inclusion of 10 nM calyculin A to the cultured cortical neurons induced neuronal apoptosis apparent by cell body shrinkage (Fig. 2e) and staining with annexin V-fluorescein isothiocyanate and propidium iodide (Fig. 2h). Calyculin A-induced apoptosis was prevented by cotreatment with 5 mM Li^+ (Fig. 2, f and i). When subtoxic doses of BAPTA-AM, the selective Ca^{2+} chelator, were included during treatment with Li^+, the neuroprotective effects of Li^+ against calyculin A were completely abrogated (Fig. 2, g, arrow, and j). The opposing effect of BAPTA-AM against Li^+ was reduced with delayed administration. In particular, when BAPTA-AM was administered to cortical cell cultures 1 h after exposure to Li^+, it did not influence antiapoptotic action of Li^+ (Fig. 2k). Thus, Li^+ seems to prevent neuronal cell apoptosis by increasing $[Ca^{2+}]_i$.

PI3-K Mediates Li^+-Induced Increase in $[Ca^{2+}]_i$ and Antiapoptosis. PI3-K acts as a key signaling molecule underlying antiapoptotic action of growth factors or depolarization and seems to mediate the neuroprotective action of Li^+ (Chalecka-Franaszek and Chuang, 1999; Crowder and Freeman, 1999; Hetman et al., 1999). Because several lines of evidence suggest that PI3-K is activated by Ca^{2+} as well as tyrosine kinases (Vaillant et al., 1999), the Li^+-induced increase in $[Ca^{2+}]_i$ may intervene in apoptosis through activation of PI3-K. In cortical cell cultures exposed to Li^+, PI3-K was activated within 30 s and maximally observed at 1 min (Fig. 3a). Activation of PI3-K remained elevated over 15 min after treatment with Li^+. PI3-K is essential for activation of the AKT/PKB serine/threonine protein kinase, an important signaling molecule known to block apoptosis. As expected, activity of AKT/PKB was increased in cortical cell cultures

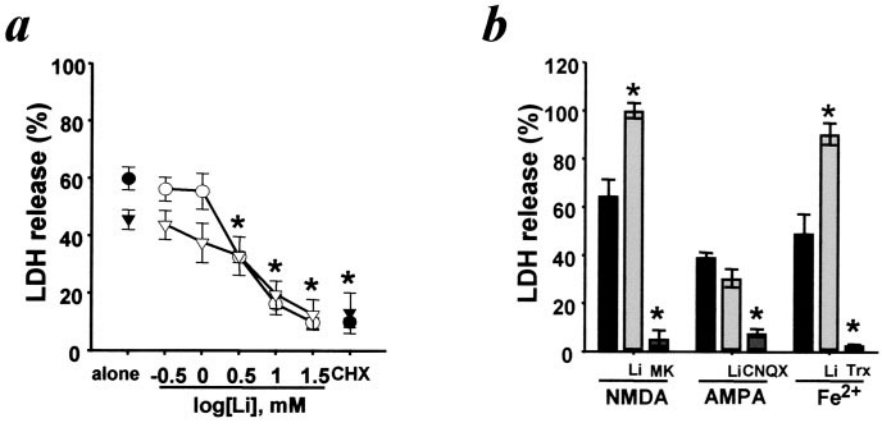

Fig. 1. Li^+ attenuates neuronal apoptosis but not necrotic cell death. a, cocultures of neurons and glia (DIV 11) were exposed to 10 nM calyculin A (circles) or 20 μM cyclosporin A (triangles), alone (solid symbols) or in the presence of indicated doses of Li^+ (open symbols) or 1 μg/ml cycloheximide (CHX). b, cultures (DIV 13–14) were exposed to the following conditions: 20 μM NMDA, alone or with 5 mM Li^+ (Li) or 10 μM MK-801 (MK); 20 μM AMPA, alone or with 5 mM Li^+ or 50 μM 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX); 50 μM Fe^{2+}, alone or with 5 mM Li^+ (Li) or 100 μM trolox (Trx). Neuronal death was analyzed 24 h later by measuring LDH release, mean ± S.E.M. (n = 8 culture wells per condition). *, $P < 0.05$, significant difference from relevant control (alone) by analysis of variance and Student-Neuman-Keuls' test.

within 5 min after administration of Li^+, which was abolished by cotreatment with a PI3-K inhibitor, wortmannin (Fig. 3b).

Concurrent treatment with 30 to 300 nM wortmannin or 3 to 30 μM LY294002, the broad inhibitors of PI3-K, reversed the neuroprotective effects of Li^+ against neuronal cell apoptosis after exposure of cortical cell cultures to calyculin A

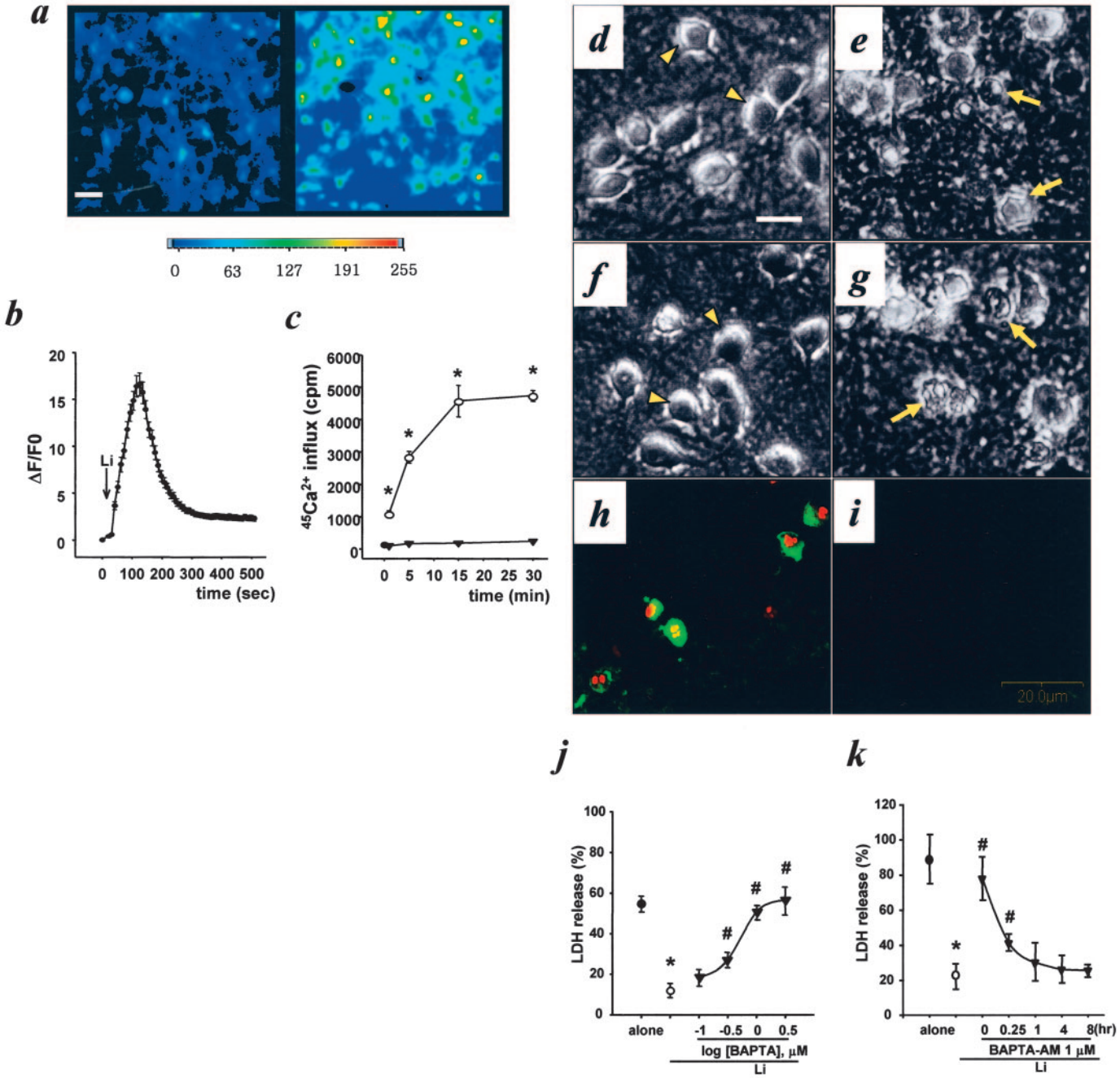

Fig. 2. Increase in $[Ca^{2+}]_i$ mediates the antiapoptosis action of Li^+. a, Fluo-3 fluorescence photomicrographs showing increased $[Ca^{2+}]_i$ in neurons immediately after exposure of cortical cell cultures (DIV 11) to a sham control (left) or 5 mM Li^+ (right) for 100 s. Scale bar, 30 μm. b, changes in neuronal $[Ca^{2+}]i$ ($\Delta F/F0$) were analyzed over 500 s after exposure of cortical cultures to 5 mM Li^+ as described under *Materials and Methods*, mean ± S.E.M. (n = 50 neurons randomly chosen from each culture). Similar results were observed from three separate experiments. c, cultures were incubated with 5 mM Li^+ (▼) or 100 μM NMDA (○) for indicated points of time in the presence of $^{45}Ca^{2+}$ (1 μCi/ml). Influx of $^{45}Ca^{2+}$ was then analyzed as described, mean ± S.E.M. (n = 8 culture wells per condition). *, significant difference from a sham control (●) at $P < 0.05$, using analysis of variance and Student-Neuman-Keuls' test. d–g, BAPTA-AM, the selective Ca^{2+} chelator, abolished the antiapoptotic effect of Li^+. Phase-contrast photomicrographs of cortical cultures 24 h after exposure to a sham control (d) or 10 nM calyculin A, alone (e) or in the presence of 5 mM Li^+ (f) or 5 mM Li^+ plus 1 μM of BAPTA-AM (g). Arrow heads and arrows indicate healthy neurons and shrunken apoptotic neurons, respectively. Scale bar, 30 μm. h and i, confocal fluorescence photomicrographs of cultured cortical neurons stained with fluorescein isothiocyanate-labeled annexin V (green) and propidium iodide (red) after 12-h exposure to 10 nM calyculin A, alone (h) or in the presence of 5 mM Li^+ (i). j and k, cortical cell cultures were exposed continuously to 10 nM calyculin A, alone (●) or in the presence of 5 mM Li^+ (○). Sister cultures were added with indicated doses of BAPTA-AM during exposure to Li^+ (j, ▼) or 1 μM BAPTA-AM at various points of time after administration of Li^+ (k, ▼). Neuronal loss was analyzed 24 h later, mean ± S.E.M. (n = 12 culture wells per condition). *, significant difference from calyculin A-treated group; #, significant difference between Li^+-treated groups with and without BAPTA-AM at $P < 0.05$, using analysis of variance and Student-Neuman-Keuls' test.

(Fig. 3, c and d). These results suggest that activation of PI3-K and increase in $[Ca^{2+}]_i$ seem to be necessary for the antiapoptotic action of Li^+. Next, we tested whether Ca^{2+} would mediate Li^+-induced activation of PI3-K. Chelating intracellular Ca^{2+} with BAPTA-AM did not interfere with activation of PI3-K after treatment with Li^+ (H. J. Kang, B. J. Gwag, unpublished observations), indicating that Ca^{2+} was not required for Li^+-induced activation of PI3-K. To the contrary, inclusion of 300 nM wortmannin completely blocked Li^+-induced increase in $[Ca^{2+}]_i$ (Fig. 3e). This raises the possibility that activation of PI3-K can induce increase in $[Ca^{2+}]_i$ that is responsible for the antiapoptosis action of Li^+.

PLCγ Mediates Ca^{2+}-Dependent Antiapoptotic Action of Li^+. It is possible that activated PI3-K by Li^+ may increase $[Ca^{2+}]_i$ through activation of PLCγ. We analyzed generation of IP_3 to determine whether Li^+ would activate PLCγ. Cortical cells challenged with Li^+ showed increased production of IP_3 up to 5- to 6-fold within 1 min (Fig. 4a). This effect of Li^+ on IP_3 production was prevented with inclusion of wortmannin. This implies that PLCγ may mediate Ca^{2+}-dependent antiapoptotic action of Li^+. In support of this, concurrent treatment with 10 μM U-73122, an inhibitor of phospholipase C, completely abrogated the antiapoptosis action of Li^+ against calyculin A (Fig. 4b). The central role of PLCγ in the neuroprotective action of Li^+ was further demonstrated in PLCγ-null fibroblasts (Ji et al., 1997). Wild-type fibroblasts deprived of serum for 72 h revealed apoptotic degeneration that was prevented by addition of 3 to 10 mM Li^+ (Fig. 4c). In PLCγ-null fibroblasts, serum deprivation-induced apoptosis was pronounced and not prevented by Li^+. Although treatment with Li^+ increased $[Ca^{2+}]_i$ rapidly in wild-type fibroblasts, no effect of Li^+ on $[Ca^{2+}]_i$ was observed in the PLCγ-null fibroblasts (Fig. 4d). Taken together, activation of PLCγ seems to be required for effects of Li^+ that elevate $[Ca^{2+}]_i$ and prevent apoptosis.

Discussion

Li^+ prevents neuronal cell apoptosis without reducing excitotoxic or oxidative neuronal necrosis. The present findings buttress an emerging hypothesis that the beneficial effects of neuroprotectants will depend upon the patterns of neuronal death. PI3-K–mediated increase in $[Ca^{2+}]_i$ is essential for antiapoptosis action of Li^+.

Apoptosis and necrosis have been adopted as major forms of neuronal death under physiological and pathological conditions. Apoptotic neurons reveal unique morphological patterns, such as shrinkage and condensation of cell body, early collapse of nuclear membrane, and aggregated condensation of nuclear chromatin (Kerr et al., 1972; Gwag et al., 1995). This apoptotic neuronal death can be induced by various neurotoxic insults, including deprivation of trophic factors,

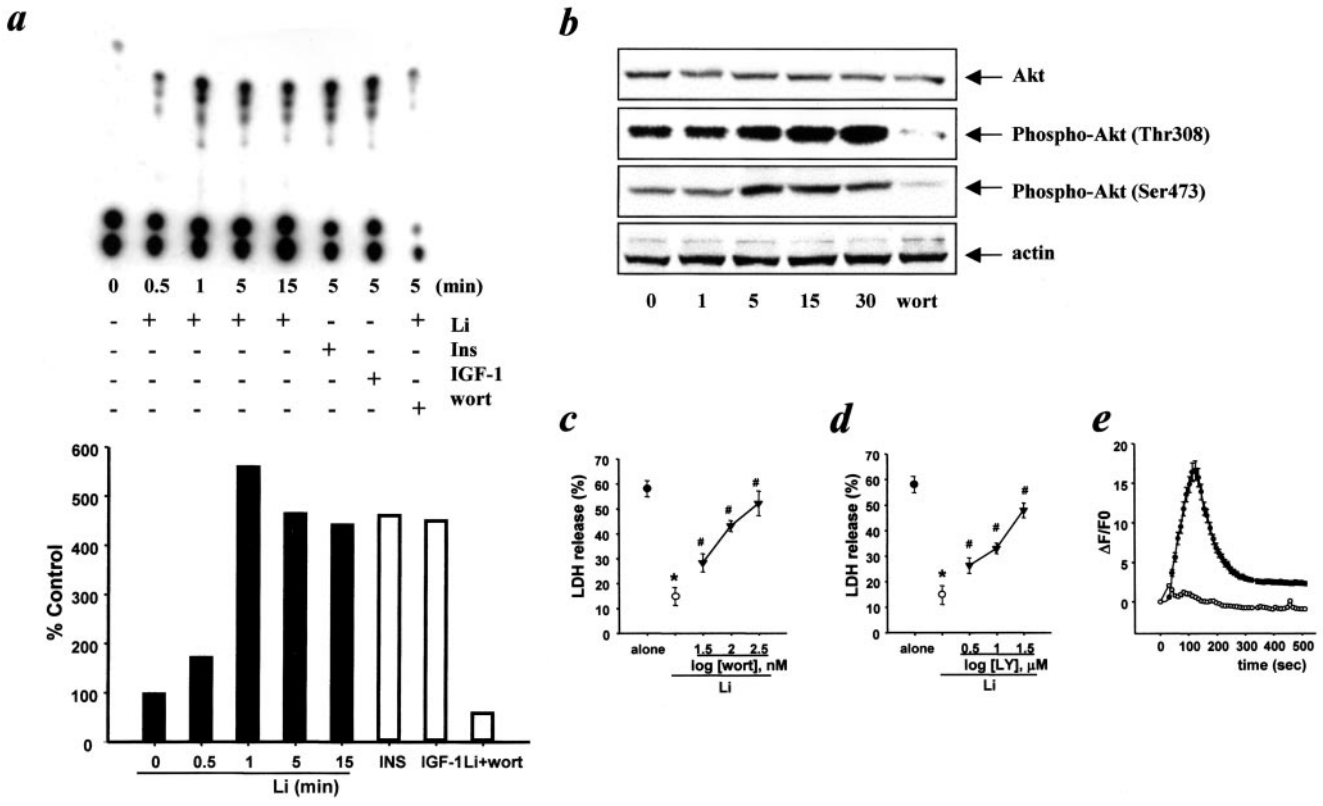

Fig. 3. PI3-K mediates Li^+-induced increase in $[Ca^{2+}]_i$. a, chromatogram (left) and its densitometry analysis (right) showing activity of PI3-K in cortical cell cultures (DIV 11) exposed to 5 mM Li^+, 100 ng/ml insulin (Ins), 100 ng/ml insulin-like growth factor-1 (IGF-1), or 5 mM Li^+ plus 100 nM wortmannin (wort) at indicated times. Similar results were obtained from three separate experiments. b, cortical cultures (DIV 11) were exposed to 5 mM Li^+ for indicated points of time (minutes) or 5 mM Li^+ plus 100 nM wortmannin for 15 min (wort). Activity of Akt kinase was analyzed by Western blot analysis using antibodies for total Akt or active forms of Akt phosphrylated at Thr308 [phospho-Akt(Thr308)] or at Ser473 [phospho-Akt(Ser473)]. An actin immunoblot was included as a loading control. c and d, cortical cell cultures were exposed to 10 nM calyculin A, alone (●) or in the presence of 5 mM Li^+ (○) or 5 mM Li^+ plus indicated doses of wortmannin (c, ▲) or LY 294002 (d, ▼). Neuronal loss was analyzed 24 h later, mean ± S.E.M. (n = 12 culture wells per condition). *, significant difference from calyculin A alone; #, significant difference between Li^+-treated groups with and without wortmannin or LY 294002 at $P < 0.05$, using analysis of variance and Student-Neuman-Keuls' test. e, changes ($\Delta F/F0$) in $[Ca^{2+}]_i$ using fluo-3 after exposure of cortical neurons to 5 mM Li^+, alone (●) or with addition of 300 nM wortmannin 15 min before Li^+ (○), mean ± S.E.M. (n = 50 neurons randomly chosen per each condition). Similar results were observed from five separate experiments.

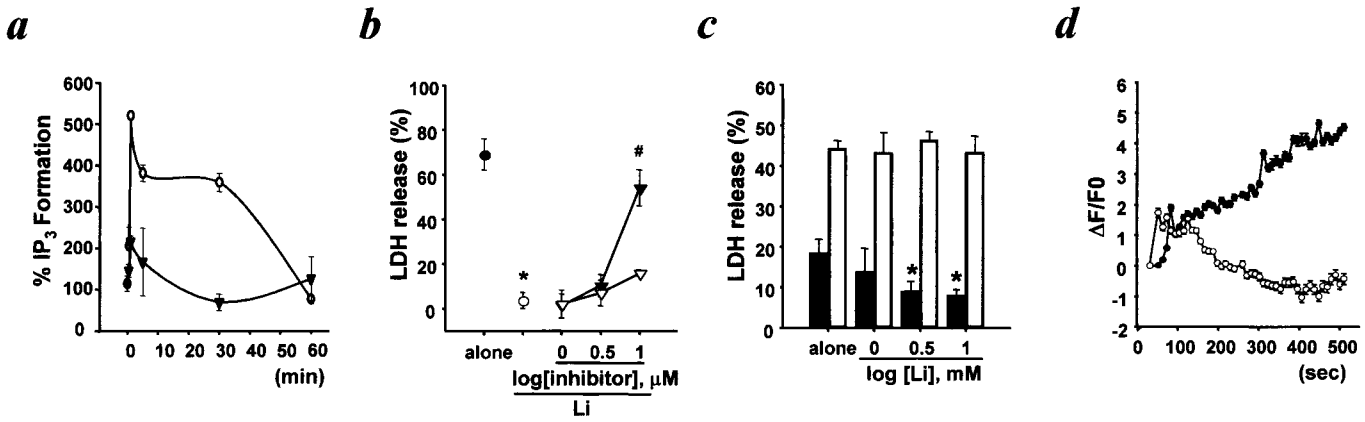

Fig. 4. PLCγ mediates Ca^{2+}-dependent antiapoptotic action of Li^+. a, cortical cell cultures (DIV 11) were exposed to 5 mM Li^+ for indicated times, alone (○) or with 300 nM wortmannin (▼). IP_3 formation was measured immediately after treatments, mean ± S.E.M. (n = 3 per condition). b, cortical cell cultures were exposed to calyculin A, alone (●) or in the presence of 5 mM Li^+ with following additions: alone (○), 5 mM Li^+ plus indicated doses of U-73122 (▼), or 5 mM Li^+ plus U-73343 (▽). Neuronal loss was analyzed 24 h later, mean ± S.E.M. (n = 12 culture wells per condition). *, significant difference from calyculin A alone; #, significant difference between Li^+-treated groups with and without U-73122 (or U-73343) at $P < 0.05$, using analysis of variance and Student-Neuman-Keuls' test. c, wild-type (■) or PLC1γ-null (□) fibroblast cells were continuously deprived of serum, alone or with 5 mM Li^+. Cell death was analyzed 3 days later by measuring efflux of LDH released into bathing media, mean ± S.E.M. (n = 12). *, significant difference from control (alone) at $P < 0.05$, using analysis of variance and Student-Neuman-Keuls' test. d, analysis of changes ($\Delta F/F0$) in $[Ca^{2+}]_i$ using fluo-3 in wild-type (●) or PLCγ1-null (○) fibroblast cells treated with 5 mM Li^+, mean ± S.E.M. (n = 30 cells randomly chosen per each condition). Similar results were observed from three separate experiments.

staurosporine, Ca^{2+} ionophores, β-amyloid fragments, or protein phosphatase inhibitors (cyclosporin A or calyculin A) and executed primarily through activation of cysteine-dependent aspartate-directed proteases (caspases) (Loo et al., 1993; Koh et al., 1995b; Deshmukh et al., 1996; McDonald et al., 1996; Gwag et al., 1999; Ko et al., 2000; Strasser et al., 2000). In contrast, neuronal necrosis is accompanied by swelling of cell body and mitochondria, early fenestration of plasma membrane, and scattering condensation of nuclear chromatin (Kerr et al., 1972; Gwag et al., 1997). These morphological patterns are observed in the process of excitotoxicity or free radical neurotoxicity and associated with overall collapse of ion homeostasis (Gwag et al., 1997; Ryu et al., 1999b). We have observed that neurotrophins, insulin-like growth factors, and gangliosides prevent neuronal apoptosis but render neurons highly vulnerable to various pronecrotic insults (Koh et al., 1995a; Ryu et al., 1999a). Furthermore, administration of glutamate antagonists blocks excitotoxic necrosis after deprivation of oxygen and glucose but unveils slowly evolving neuronal apoptosis (Gwag et al., 1995). These findings lead us to hypothesize that the neuroprotective action of Li^+ as well as other neuroprotectants may depend upon the patterns (apoptosis versus necrosis) of death. In support of this, Li^+ prevents neuronal cell apoptosis by calyculin A, cyclosporin A, or serum deprivation but does not show beneficial effects against necrosis by excitotoxicity or oxidative stress.

Administration of large amounts of potassium depolarizes neurons and elevates $[Ca^{2+}]_i$ through activation of voltage-gated Ca^{2+} channels, which promotes neuronal survival by blocking apoptosis (Franklin et al., 1995). These findings have raised a novel hypothesis that neurons can be rescued from apoptosis in the presence of appropriate $[Ca^{2+}]_i$ or trophic factors. We found that cortical neurons treated with Li^+ revealed rapid and sustained increase in $[Ca^{2+}]_i$. Concurrent inclusion of the selective Ca^{2+} chelator BAPTA-AM completely abrogated antiapoptotic effects of Li^+. This implies that raised $[Ca^{2+}]_i$ mediates the antiapoptosis action of Li^+. Ca^{2+}-dependent neuroprotective action seems to be unique to Li^+ in that BAPTA-AM does not influence the neuroprotective effects of brain-derived neurotrophic factor or insulin against the same apoptosis-inducing agents (H. J. Kang, B. J. Gwag, unpublished data). The present study demonstrates that Li^+ prevents neuronal apoptosis by increasing $[Ca^{2+}]_i$.

Growth factors such as nerve growth factor and insulin-like growth factors activate PI3-K through the receptor tyrosine kinase pathway (Yao and Cooper, 1995). Activated PI3-K generates D3-phosphorylated phosphoinositides that activate the phosphoinositide-dependent kinase-1 (PDK1) and thus induce the phosphorylation at Thr308 of the serine-threonine protein kinase Akt (Anderson et al., 1998). Active Akt can phosphorylate a proapoptotic Bcl-2 family member, BAD, and a cysteine protease, caspase-9, that inhibit execution of apoptosis (Datta et al., 1997; Khwaja, 1999).

Whereas PI3-K–PDK1–Akt is an essential pathway for antiapoptosis action of growth factors irrespective of $[Ca^{2+}]_i$ (Yao and Cooper, 1995; Hetman et al., 1999), the present findings suggest that Ca^{2+} can act as a downstream signal of PI3-K essential for antiapoptosis action of Li^+. First, BAPTA-AM completely blocked the antiapoptotic effects of Li^+ but did not interfere with activation of PI3-K by Li^+. This suggests that activation of PI3-K precedes increase in $[Ca^{2+}]_i$ after exposure to Li^+. Second, Li^+-induced increase in $[Ca^{2+}]_i$ was prevented by inhibitors of PI3-K that reversed the antiapoptotic effects of Li^+. Increased $[Ca^{2+}]_i$ through activation of PI3-K can interfere with propagation of apoptosis by phosphorylation of Thr308 of Akt through activation of Ca^{2+}/calmodulin-dependent protein kinase kinase (Yano et al., 1998; Chalecka-Franaszek and Chuang, 1999).

Phosphatydylinositol 3,4,5-triphosphate, the lipid product of PI3-K, binds to the *Src* homology domain 2 or the pleckstrin homology domain of PLCγ, which results in enhanced activation of PLCγ and increase in $[Ca^{2+}]_i$ (Bae et al., 1998; Falasca et al., 1998). In the present study, administration of Li^+ results in accumulation of intracellular Ca^{2+} without influencing influx of extracellular Ca^{2+}. Exposure of cortical

cell cultures to Li^+ reveals increased IP_3 production that disappears in the presence of PI3-K inhibitors. U-73122, a selective inhibitor of phospholipase C, or gene targeting of PLCγ blocks the antiapoptosis action of Li^+. Treatment with Li^+ does not increase $[Ca^{2+}]_i$ in PLCγ-null fibroblasts. Taken together, Li^+ seems to increase $[Ca^{2+}]_i$ through PI3-K-mediated activation of PLCγ.

The mood-stabilizing actions of Li^+ are primarily attributed to inhibition of inositol monophosphatase and inositol polyphosphate 1-phosphatase that interrupts recycling of inositol, results in depletion of inositol, and eventually reduces the generation of IP_3 through various receptors (Berridge et al., 1989; Casebolt and Jope, 1989; Song and Jope, 1992; Dixon et al., 1994). In contrast to long-term effects of Li^+ leading to depletion of IP_3, the present study demonstrates that administration of Li^+ results in rapid and sustained accumulation of intracellular Ca^{2+}. The Li^+-induced increase in $[Ca^{2+}]_i$ seems to be derived from internal Ca^{2+} stores that depend upon activation of PI3-K and PLCγ. These signaling events of Li^+ underlie beneficial effects against apoptosis and probably hold therapeutic promise to intervene in neuronal apoptosis evolving after acute brain injuries, such as hypoxic ischemia, trauma, and epilepsy.

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References

Anderson KE, Coadwell J, Stephens LR, and Hawkins PT (1998) Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B. *Curr Biol* **8:**684–691.

Bae YS, Cantley LG, Chen CS, Kim SR, Kwon KS, and Rhee SG (1998) Activation of phospholipase C-γ by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* **273:** 4465–4469.

Berridge MJ, Downes CP, and Hanley MR (1989) Neural and developmental actions of lithium: a unifying hypothesis. *Cell* **59:**411–419.

Bijur GN, De Sarno P, and Jope RS (2000) Glycogen synthase kinase-3beta facilitates staurosporine- and heat shock-induced apoptosis. Protection by lithium. *J Biol Chem* **275:**7583–7590.

Casebolt TL and Jope RS (1989) Long-term lithium treatment selectively reduces receptor-coupled inositol phospholipid hydrolysis in rat brain. *Biol Psychiatry* **25:**329–340.

Centeno F, Mora A, Fuentes JM, Soler G, and Claro E (1998) Partial lithium-associated protection against apoptosis induced by C2-ceramide in cerebellar granule neurons. *Neuroreport* **9:**4199–4203.

Chalecka-Franaszek E and Chuang DM (1999) Lithium activates the serine/threonine kinase Akt-1 and suppresses glutamate-induced inhibition of Akt-1 activity in neurons. *Proc Natl Acad Sci USA* **96:**8745–8750.

Choi DW (1996) Ischemia-induced neuronal apoptosis. *Curr Opin Neurobiol* **6:**667–672.

Cimarosti H, Rodnight R, Tavares A, Paiva R, Valentim L, Rocha E, and Salbego C (2001) An investigation of the neuroprotective effect of lithium in organotypic slice cultures of rat hippocampus exposed to oxygen and glucose deprivation. *Neurosci Lett* **315:**33–36.

Crowder RJ and Freeman RS (1999) The survival of sympathetic neurons promoted by potassium depolarization, but not by cyclic AMP, requires phosphatidylinositol 3-kinase and Akt. *J Neurochem* **73:**466–475.

D'Mello SR, Anelli R, and Calissano P (1994) Lithium induces apoptosis in immature cerebellar granule cells but promotes survival of mature neurons. *Exp Cell Res* **211:**332–338.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, and Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91:**231–241.

Deshmukh M, Vasilakos J, Deckwerth TL, Lampe PA, Shivers BD, and Johnson EMJ (1996) Genetic and metabolic status of NGF-deprived sympathetic neurons saved by an inhibitor of ICE family proteases. *J Cell Biol* **135:**1341–1354.

Dixon JF, Los GV, and Hokin LE (1994) Lithium stimulates glutamate "release" and inositol 1,4,5-trisphosphate accumulation via activation of the *N*-methyl-D-aspartate receptor in monkey and mouse cerebral cortex slices. *Proc Natl Acad Sci USA* **91:**8358–8362.

Dixon JF and Hokin LE (1998) Lithium acutely inhibits and chronically up-regulates and stabilizes glutamate uptake by presynaptic nerve endings in mouse cerebral cortex. *Proc Natl Acad Sci USA* **95:**8363–8368.

Falasca M, Logan SK, Lehto VP, Baccante G, Lemmon MA, and Schlessinger J (1998) Activation of phospholipase C gamma by PI 3-kinase-induced PH domain-mediated membrane targeting. *EMBO (Eur Mol Biol Organ) J* **17:**414–422.

Franklin JL and Johnson EMJ (1994) Elevated intracellular calcium blocks programmed neuronal death. *Ann NY Acad Sci* **747:**195–204.

Franklin JL, Sanz-Rodriguez C, Juhasz A, Deckwerth TL, and Johnson EMJ (1995) Chronic depolarization prevents programmed death of sympathetic neurons in vitro but does not support growth: requirement for Ca^{2+} influx but not Trk activation. *J Neurosci* **15:**643–664.

Goodwin FK and Jamison KR (1990) *Manic-Depressive Illness*, Oxford University Press, New York.

Gottron FJ, Ying HS, and Choi DW (1997) Caspase inhibition selectively reduces the apoptotic component of oxygen-glucose deprivation-induced cortical neuronal cell death. *Mol Cell Neurosci* **9:**159–169.

Gwag BJ, Lobner D, Koh JY, Wie MB, and Choi DW (1995) Blockade of glutamate receptors unmasks neuronal apoptosis after oxygen-glucose deprivation in vitro. *Neuroscience* **68:**615–619.

Gwag BJ, Koh JY, DeMaro JA, Ying HS, Jacquin M, and Choi DW (1997) Slowly triggered excitotoxicity occurs by necrosis in cortical cultures. *Neuroscience* **77:** 393–401.

Gwag BJ, Canzoniero LM, Sensi SL, Demaro JA, Koh JY, Goldberg MP, Jacquin M, and Choi DW (1999) Calcium ionophores can induce either apoptosis or necrosis in cultured cortical neurons. *Neuroscience* **90:**1339–1348.

Hallcher LM and Sherman WR (1980) The effects of lithium ion and other agents on the activity of myo-inositol-1-phosphatase from bovine brain. *J Biol Chem* **255:** 10896–10901.

Hetman M, Kanning K, Cavanaugh JE, and Xia Z (1999) Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *J Biol Chem* **274:**22569–22580.

Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, and Woodgett JR (2000) Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature (Lond)* **406:**86–90.

Hong M, Chen DC, Klein PS, and Lee VM (1997) Lithium reduces tau phosphorylation by inhibition of glycogen synthase kinase-3. *J Biol Chem* **272:**25326–25332.

Ji QS, Winnier GE, Niswender KD, Horstman D, Wisdom R, Magnuson MA, and Carpenter G (1997) Essential role of the tyrosine kinase substrate phospholipase C-gamma1 in mammalian growth and development. *Proc Natl Acad Sci USA* **94:**2999–3003.

Kao JP, Harootunian AT, and Tsien RY (1989) Photochemically generated cytosolic calcium pulses and their detection by fluo-3. *J Biol Chem* **264:**8179–8184.

Kerr JF, Wyllie AH, and Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26:**239–257.

Khwaja A (1999) Akt is more than just a Bad kinase. *Nature (Lond)* **401:**33–34.

Ko HW, Han KS, Kim EY, Ryu BR, Yoon WJ, Jung YK, Kim SU, and Gwag BJ (2000) Synergetic activation of p38 mitogen-activated protein kinase and caspase-3-like proteases for execution of calyculin A-induced apoptosis but not *N*-methyl-D-aspartate-induced necrosis in mouse cortical neurons. *J Neurochem* **74:**2455–2461.

Koh JY and Choi DW (1987) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J Neurosci Methods* **20:**83–90.

Koh JY, Gwag BJ, Lobner D, and Choi DW (1995a) Potentiated necrosis of cultured cortical neurons by neurotrophins. *Science (Wash DC)* **268:**573–575.

Koh JY, Wie MB, Gwag BJ, Sensi SL, Canzoniero LM, Demaro J, Csernansky C, and Choi DW (1995b) Staurosporine-induced neuronal apoptosis. *Exp Neurol* **135:**153–159.

Loo DT, Copani A, Pike CJ, Whittemore ER, Walencewicz AJ, and Cotman CW (1993) Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. *Proc Natl Acad Sci USA* **90:**7951–7955.

Manji HK, Potter WZ, and Lenox RH (1995) Signal transduction pathways. Molecular targets for lithium's actions. *Arch Gen Psychiatry* **52:**531–543.

McDonald JW, Goldberg MP, Gwag BJ, Chi SI, and Choi DW (1996) Cyclosporine induces neuronal apoptosis and selective oligodendrocyte death in cortical cultures. *Ann Neurol* **40:**750–758.

Nonaka S, Hough CJ, and Chuang DM (1998a) Chronic lithium treatment robustly protects neurons in the central nervous system against excitotoxicity by inhibiting *N*-methyl-D-aspartate receptor-mediated calcium influx. *Proc Natl Acad Sci USA* **95:**2642–2647.

Nonaka S, Katsube N, and Chuang DM (1998b) Lithium protects rat cerebellar granule cells against apoptosis induced by anticonvulsants, phenytoin and carbamazepine. *J Pharmacol Exp Ther* **286:**539–547.

Ryu BR, Choi DW, Hartley DM, Costa E, Jou I, and Gwag BJ (1999a) Attenuation of cortical neuronal apoptosis by gangliosides. *J Pharmacol Exp Ther* **290:**811–816.

Ryu BR, Ko HW, Jou I, Noh JS, and Gwag BJ (1999b) Phosphatidylinositol 3-kinase-mediated regulation of neuronal apoptosis and necrosis by insulin and IGF-I. *J Neurobiol* **39:**536–546.

Song L and Jope RS (1992) Chronic lithium treatment impairs phosphatidylinositol hydrolysis in membranes from rat brain regions. *J Neurochem* **58:**2200–2206.

Strasser A, O'Connor L, and Dixit VM (2000) Apoptosis signaling. *Annu Rev Biochem* **69:**217–245.

Vaillant AR, Mazzoni I, Tudan C, Boudreau M, Kaplan DR, and Miller FD (1999) Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. *J Cell Biol* **146:**955–966.

Wei H, Qin ZH, Senatorov VV, Wei W, Wang Y, Qian Y, and Chuang DM (2001) Lithium suppresses excitotoxicity-induced striatal lesions in a rat model of Huntington's disease. *Neuroscience* **106:**603–612.

Yano S, Tokumitsu H, and Soderling TR (1998) Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature (Lond)* **396:**584–587.

Yao R and Cooper GM (1995) Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science (Wash DC)* **267:**2003–2006.

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