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β-Amyloid peptide-induced death of PC 12 cells and cerebellar granule cell neurons is inhibited by long-term lithium treatment

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Abstract

Treatment of rat pheochromocytoma cells (PC 12) cells with β-amyloid peptide-(1–42) for 24 h induced a concentration-dependent decrease in cellular redox activity in the dose range of 1 to 20 μM. These effects were markedly attenuated by pretreatment with 2 mM LiCl for 7 days, whereas 1-day pretreatment was ineffective. Measurements of live and dead cells by double-staining with fluorescein diacetate and propidium iodide, respectively revealed that protracted lithium pretreatment attenuated PC 12 cell death induced by β-amyloid-(1–42) and cerebellar granule cell death induced by β-amyloid-(25–35). Preceding PC 12 cell death, β-amyloid peptide elicited a slight decrease in protein levels of Bcl-2. Conversely, 7-day pretreatment with lithium resulted in an approximate doubling of Bcl-2 protein levels in cells treated with or without β-amyloid peptide-(1–42). Lithium-induced Bcl-2 upregulation was temporally associated with the cytoprotective effects of this drug. Thus, lithium protection against β-amyloid peptide neurotoxicity might involve Bcl-2 overexpression, and lithium treatment for Alzheimer's disease should be reexamined.

Keywords: Lithium chloride; β-Amyloid; Bcl-2; Alzheimer's disease

1. Introduction

Amyloid β-peptide is a 40–42 amino acid peptide that accumulates as insoluble extracellular deposits in senile plaques of Alzheimer's disease patients (for review, see Selkoe, 1991). Several lines of evidence support that β-amyloid peptide may play an important role in the pathogenesis of Alzheimer's disease. β-Amyloid peptide is proteolytically derived from a larger β-amyloid precursor protein and mutations of this precursor protein around the β-amyloid peptide domain are linked to inherited Alzheimer's disease (Tanzi et al., 1993; Price et al., 1998). In cultures, β-amyloid peptide can directly induce neuronal cell death and can render neurons vulnerable to excitotoxicity (Koh et al., 1990) and oxidative insults (Mattson et al., 1993; Goodman and Mattson, 1994). The mechanisms underlying β-amyloid peptide neurotoxicity are complex but may involve disruption of intracellular homeostasis of Ca^{2+} (Mattson et al., 1993) and potassium (Colom et al., 1998), induction of oxidative stress (Behl et al., 1992; Goodman and Mattson, 1994; Tomiyama et al., 1996) as well as upregulation of proapoptotic Bax simultaneously with downregulation of neuroprotective Bcl-2 (Paradis et al., 1996).

In the search for a novel protective agent against β-amyloid peptide-induced neurotoxicity, we have examined the effects of lithium, the drug most commonly used to treat manic depressive illness. We have recently demonstrated that long-term lithium treatment robustly protects CNS neurons from glutamate-induced excitotoxicity and that this neuroprotection is due, at least in part, to suppression of *N*-methyl-D-aspartate (NMDA)-receptor-mediated Ca^{2+} influx (Nonaka et al., 1998a), and upregulation of Bcl-2 with simultaneous downregulation of Bax and p53 (Chen and Chuang, 1999). Other studies show that lithium reduces apoptotic death induced by growth factor with-

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drawal (Volonté and Rukenstein, 1993), KCl deprivation (Grignon et al., 1996), anticonvulsant treatment (Nonaka et al., 1998b), and thapsigargin-induced Ca^{2+} depletion from the endoplasmic reticulum (Wei et al., 1998). In vivo, chronic lithium administration to rats reduces focal cerebral ischemia-induced brain infarction (Nonaka and Chuang, 1998). Given the potential mechanisms of β-amyloid peptide-induced neurotoxicity, it seems reasonable to explore the neuroprotective effects of lithium against β-amyloid peptide-induced apoptotic death and to study associated potential underlying mechanisms. Preliminary results of this study have been presented in an abstract form (Wei et al., 1999).

2. Materials and methods

2.1. Materials

RPMI 1640 medium, horse serum, fetal calf serum and gentamicin were purchased from Gibco BRL (Gaithersburg, MD). Primary monoclonal antibody against Bcl-2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibody and enhanced chemiluminescent detection kit for Western blotting were from Amersham Life Science (Piscataway, NJ). All other chemicals were obtained from Sigma(St. Louis, MO).

2.2. Cell culture

Rat pheochromocytoma cells (PC 12) were maintained in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal calf serum and gentamicin (50 μg/ml). Monolayer cultures at a density of $(0.1 \text{ to } 0.3) \times 10^6$ cells/cm^2 were incubated in plastic flasks precoated with 0.03% poly-L-ornithine in a 95% air, 5% CO_2 humidified atmosphere at 37°C. The culture medium was changed every 48 h.

Cerebellar granule cell neurons were prepared from 8-day-old Sprague–Dawley rats as described (Nonaka et al., 1998a,b). The cells were maintained in basal modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, gentamicin (50 μg/ml), and 25 mM KCl. The cells were plated at a density of 0.75×10^6 cells per well in a 24-well plate precoated with poly-L-lysine. Cytosine arabinoside (10 μM) was added to the cultures 24 h after plating to arrest the growth of non-neuronal cells.

2.3. Measurement of cell viability

The mitochondrial dehydrogenase activity that reduces 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was used to determine cellular redox activity, an initial indicator of cell death, in a quantitative colorimetric assay (Nonaka et al., 1998a). PC 12 cells cultured in 96-well plates at a density of 5000 cells/well were exposed to β-amyloid peptide-(1–42) at various concentrations for 24 h, with or without 2 mM lithium pretreatment. Thereafter, PC 12 cells were incubated with MTT (125 μg/ml) added to the growth medium for 1 h at 37°C. The medium was then aspirated and the MTT reduction product, formazan, was dissolved in dimethyl sulfoxide and quantified spectrophotometrically at 540 nm. MTT reduction was expressed as percentage of control ± S.E.M. from at least three independent experiments.

Cell viability was also examined by staining with fluorescein diacetate and propidium iodide for live and dead cells, respectively, essentially as reported (Ishitani et al., 1996). Fluorescein diacetate diffuses into the cells and is cleaved by esterases present in live cells, producing yellowish green fluorescein, while propidium iodide passes through the plasma membranes of dead cells to bind to DNA, yielding orange-brown nuclei. PC 12 cells and cerebellar granule cells grown on 24-well plates were treated with β-amyloid peptides in the absence or presence of lithium pretreatment as indicated. Fluorescein diacetate (3.76 μg in 0.4% acetone/phosphate-buffered saline) and propidium iodide (1.16 μg) were added to each well containing 0.5 ml of culture medium. Live and dead cells were immediately photographed using a Zeiss Axiovert S 100 TV fluorescence microscope with 480 and 546 nm filters. Cell viability was determined by the ratio of the number of fluorescein diacetate/fluorescein diacetate + propidium iodide stained positive cells in at least four representative fields in independent cultures.

2.4. Western blotting

PC 12 cells grown on 10-cm dishes were treated with 5 μM β-amyloid peptide-(1–42). Cells were detached by scraping and sonicated for 30 s in lysis buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA (pH 8), 0.2 mM sodium orthovanadate, 0.2 mM *p*-methyl sulfonylfluoride, 0.5% NP-40, 5 μg/ml leupetin, and 5 μg/ml aprotinin. Amounts of 100 μg of total protein were separated by 4–20% Tris-glycine gel electrophoresis, and proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The blots were incubated with a monoclonal antibody against Bcl-2, and then probed with a horseradish peroxidase-conjugated secondary antibody. Detection was performed by using enhanced chemiluminescence and photographed. For quantification of Western blots, densitometry was performed by capturing images on film using a closed circuit digital camera (Sierra Scientific, Sunnyvale, CA) using Macintosh NIH image 1.5 software (Wayne Rasband, NIMH, Bethesda, MD). Details of the experimental procedures are as described (Chen and Chuang, 1999) with some modifications.

3. Results

Exposure of PC 12 cells to β-amyloid peptide-(1–42) for 24 h induced a concentration-dependent loss of MTT reducing activity with an EC_{50} value of approximately 5 μM (Fig. 1). Pretreatment of cells with 2 mM LiCl for 1 day did not significantly alter the degree of β-amyloid peptide-induced loss of MTT reduction. In contrast, pretreatment with LiCl for 7 days markedly suppressed β-amyloid peptide-induced loss of redox activity at all concentrations of β-amyloid peptide-(1–42) examined. The MTT reducing values expressed as percent of untreated controls in the absence versus presence of lithium pretreatment in cells treated with 1, 5 and 10 μM β-amyloid peptide-(1–42) were 73 ± 1.9% versus 112 ± 1.5%, 62 ± 1.8% versus 91 ± 2.6%, and 42.8 ± 6.3% versus 78 ± 12%, respectively. In untreated cells, lithium also slightly increased the MTT reducing activity

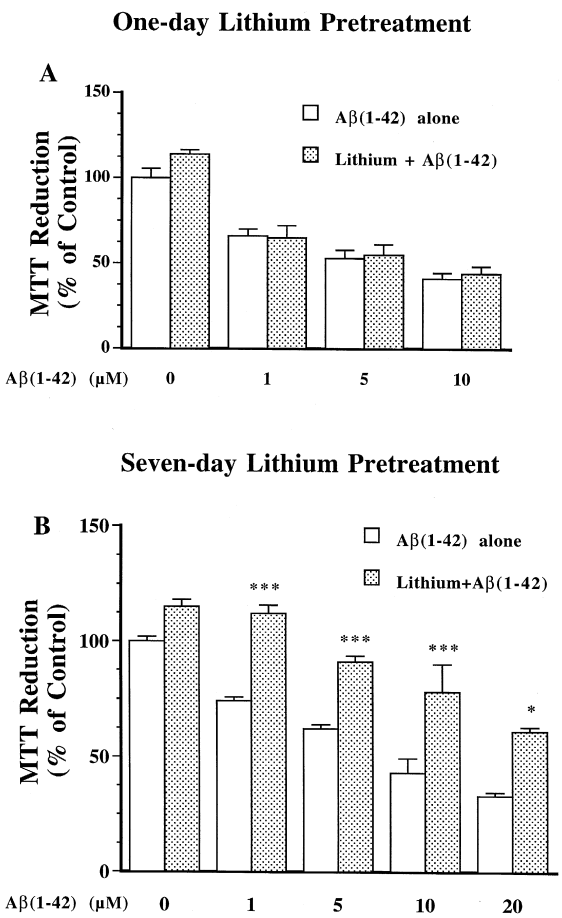


Fig. 1. Long-term lithium treatment inhibits β-amyloid peptide-(1–42)-induced loss of redox actovity in PC 12 cells. PC 12 cells cultured in 96-well plates were pretreated with 2 mM LiCl for 1 day (A) or 7 days (B). Cells were then exposed to various concentrations of β-amyloid peptide-(1–42) for 24 h. Cellular redox activity was then determined by MTT analysis and was expressed as mean ± S.E.M. of untreated control from three to four independent experiments. For statistical analysis, ANOVA was performed followed by Student–Newman–Keuls multiple comparisons test. * and *** represent $p < 0.05$ and $p < 0.001$, respectively, compared to corresponding group treated with β-amyloid peptide-(1–42) alone.

A direct measure of cell death was performed using fluorescein diacetate and propidium iodide to label live and dead cells, respectively. Exposure of PC 12 cells to 10 μM β-amyloid peptide-(1–42) for 4 days increased the number of dead cells and therefore decreased cell viability compared with the untreated control (Fig. 2A versus C; Fig. 3A). One- or 2-day treatment with β-amyloid peptide-(1–42) did not significantly affect cell viability (data not shown). This β-amyloid peptide-induced PC 12 cell death was markedly suppressed by pretreatment with 2 mM LiCl for 3 days followed by its continuous presence during β-amyloid peptide-(1–42) treatment (Fig. 2D; Fig. 3A). Exposure of cerebellar granule cell neurons to 20 μM β-amyloid peptide-(25–35) for 4 days also markedly enhanced neuronal death. This β-amyloid peptide-induced neurotoxicity was also inhibited by pretreatment with 3 mM LiCl for 24 h followed by its continuous presence during β-amyloid peptide-(25–35) exposure (Fig. 2E, G and H; Fig. 3B). In both PC 12 and cerebellar granule cells, long-term lithium pretreatment alone did not substantially affect cell viability (Fig. 2B and F; Fig. 3A and B).

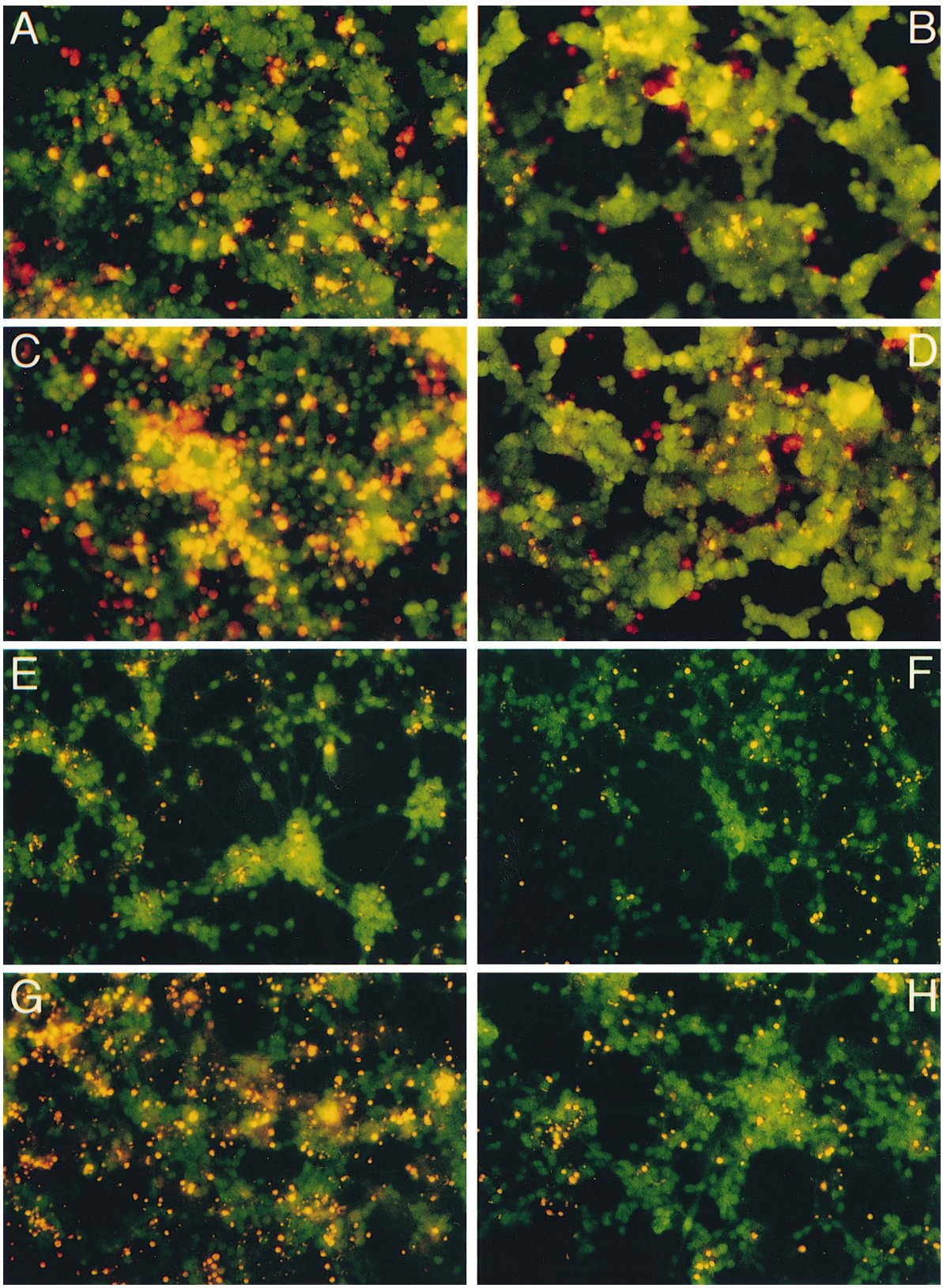
Lithium also protected PC12 cells from toxicity induced by treatment with the β-amyloid peptide-(25–35) fragment (results not shown). Moreover, lithium reduced excitotoxicity in PC 12 cells induced by high concentrations of glutamate in the presence of glycine, an NMDA receptor co-agonist (data not shown). However, MK-801, an NMDA receptor antagonist, failed to affect β-amyloid peptide-(1–42) or β-amyloid peptide-(25–35)-induced PC 12 cell death (data not shown).

Potential mechanisms underlying lithium's protection was investigated by the measurement of levels of Bcl-2 protein, a major cytoprotective gene product. Treatment of PC 12 cells with 2 mM LiCl induced a time-dependent increase in Bcl-2 protein levels determined by Western blotting analysis (Fig. 4A). The increase was insignificant at day 1, but became evident at days 3, 5 and 7. A 7-day treatment elicited an approximate twofold increase in Bcl-2 protein levels (Fig. 4B). In contrast, exposure of cells to 5 μM β-amyloid peptide-(1–42) for 6 h produced a slight decrease in Bcl-2 protein levels. Importantly, lithium pretreatment maintained Bcl-2 levels in an elevated state in cells exposed to β-amyloid peptide-(1–42).

4. Discussion

PC 12 pheochromocytoma cell line was chosen for the present study because this nerve cell type is more vulnerable to β-amyloid peptide insult and has been used extensively to study β-amyloid peptide neurotoxicity. It has been reported that inhibition of cell redox activity determined by MTT assays is an early indicator of the mecha-

nism of PC 12 cell death induced by β-amyloid peptide and this effect is followed by a delayed reduction in cell survival (Shearman et al., 1994). Confirming this observation, we found that exposure of PC 12 cells to β-amyloid peptide-(1–42) for 24 h induced a concentration-dependent loss of MTT reducing activity. Pretreatment of cells with 2



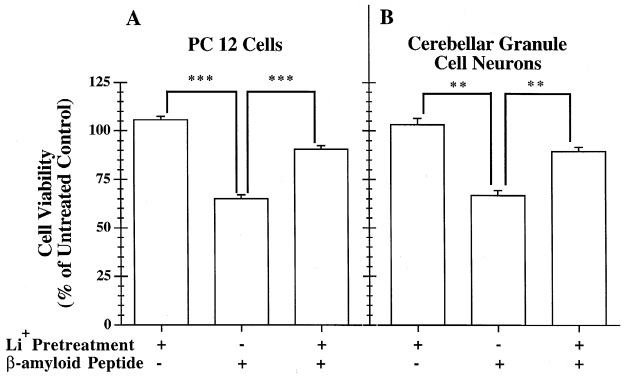


Fig. 3. Quantitative analysis of viability of cells treated with lithium and/or β-amyloid peptide. Experimental conditions of lithium and β-amyloid peptide treatments are as described in the legend to Fig. 2. In (A), PC 12 cells were treated with 2 mM LiCl beginning on the second day after plating, followed by 10 μM β-amyloid peptide-(1–42) on the fifth day in culture. Cell viability was examined on the ninth day in culture. In (B), cerebellar granule cell neurons were treated with 3 mM LiCl at 24 h after plating, followed by addition of 20 μM β-amyloid peptide-(25–35) on the fourth day in culture. Cell viability was examined on the eighth day in culture. Viability of PC 12 cells and cerebellar granule cells was determined by staining with fluorescein diacetate and propidium iodide as described in the methods and expressed as mean ± S.E.M. of the number of fluorescein diacetate/fluorescein + propidium iodide positive cells in at least four representative fields from three to four independent cultures. ** and *** represent $p < 0.01$ and $p < 0.001$ compared to corresponding control. The numbers of independent experiments are four in (A) and three in (B).

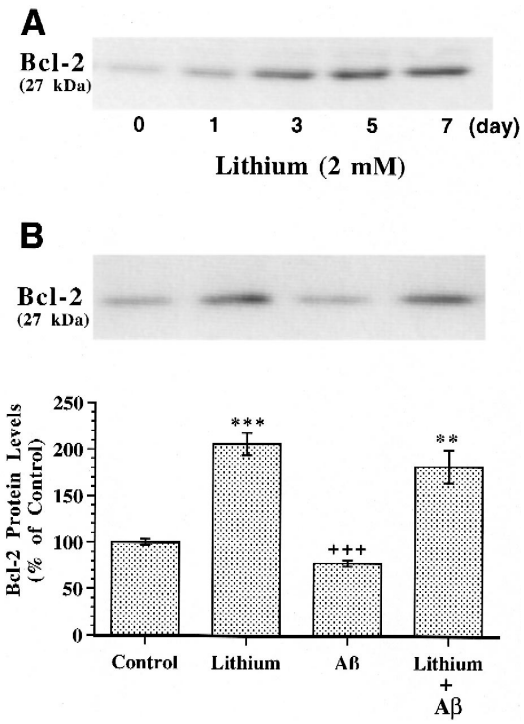


Fig. 4. Long-term lithium treatment increases Bcl-2 protein levels and suppresses β-amyloid peptide-induced loss of Bcl-2 protein in PC12 cells. (A) Cells were treated with 2 mM LiCl for indicated time periods. (B) Cells were treated with 5 μM β-amyloid peptide-(1–42) for 6 h with or without pretreatment of 2 mM LiCl for 7 days. Cells in the control group received no treatment. Bcl-2 protein levels were determined by Western blotting as described in the methods. The Western blot shown was derived from a typical experiment. Similar results were obtained from three independent experiments. Quantified results are the means ± S.E.M. from three independent experiments. ** and *** represent $p < 0.01$ and $p < 0.001$, respectively, compared to control. + + + represents $p < 0.001$ compared to the group of lithium + β-amyloid peptide as determined by ANOVA followed by Student–Newman–Keuls multiple comparisons test.

mM LiCl for 1 day did not significantly alter the degree of β-amyloid peptide-induced loss of MTT reduction. However, pretreatment with LiCl for 7 days did markedly suppress β-amyloid peptide-induced loss of redox activity at all concentrations of β-amyloid peptide-(1–42) examined, indicating a protective effect of lithium against β-amyloid peptide-(1–42)-induced toxicity. In untreated cells, lithium also slightly increased the MTT reducing activity, consistent with the trophic action of lithium in PC 12 cells (Volonté and Rukenstein, 1993).

We then directly measured cell death using fluorescein diacetate and propidium iodide to label live and dead cells, respectively. Exposure of PC 12 cells or cerebellar granule cell neurons to β-amyloid peptide for 4 days decreased cell viability compared with their respective untreated controls (Figs. 2 and 3), supporting the previous report that β-amyloid peptide-induced PC 12 cell death is a delayed event (Shearman et al., 1994). Importantly, this β-amyloid peptide-induced death of PC 12 cells and cerebellar granule cell neurons was markedly suppressed by pretreatment with LiCl followed by its continuous presence during β-amyloid peptide treatment. Thus, our results demonstrate the neuroprotective effects of lithium against β-amyloid peptide-induced cell death in cultured brain neurons and neurally related cell types.

In an attempt to elucidate potential mechanisms underlying lithium's protection, we measured the protein levels of Bcl-2 under our experimental conditions. Treatment of PC 12 cells with 2 mM LiCl induced a time-dependent

Fig. 2. Lithium pretreatment reduces β-amyloid peptide-induced death of PC 12 cells and cerebellar granule cell neurons. PC 12 cells seeded onto 24-well plates were treated with 2 mM LiCl beginning on the second day after plating, followed by 10 μM β-amyloid peptide-(1–42) on the fifth day in culture. Lithium was continuously present during treatment with β-amyloid peptide. Fluorescein diacetate/propidium iodide double-staining for live and dead cells was performed on the ninth day in culture. (A) Untreated control; (B) lithium pretreatment alone; (C) β-amyloid peptide-(1–42) alone; (D) lithium pretreatment + β-amyloid peptide-(1–42). Cerebellar granule cells were treated with 3 mM LiCl at 24 h after plating, followed by addition of 20 μM β-amyloid peptide-(25–35) on the fourth day in culture. Fluorescein diacetate/propidium iodide double-taining was performed on the eighth day in culture. (E) untreated control; (F) lithium pretreatment alone; (G) β-amyloid peptide-(25–35) alone; (H) lithium pretreatment + β-amyloid peptide-(25–35). Photographs were taken at 200 × magnification.

increase in Bcl-2 protein levels determined by Western blotting analysis with a twofold increase in Bcl-2 protein levels after 7-day treatment. Interestingly, exposure of cells to 5 μM β-amyloid peptide-(1–42) for 6 h produced a slight decrease in Bcl-2 protein levels, an effect similar to that found in human primary neurons (Paradis et al., 1996). Moreover, Bcl-2 levels were maintained in an elevated state by lithium pretreatment in cells exposed to β-amyloid peptide-(1–42). These results suggest a potential role of Bcl-2 in mediating the neuroprotective action. This notion is inconsistent with the report that transfection of the Bcl-2 gene into PC 12 cells fails to prevent the toxic effect of β-amyloid peptide-(25–35) (Behl et al., 1993). It is conceivable that additional lithium-induced effects contribute to the lithium protection. For example, lithium has been shown to markedly decrease the levels of p53 and Bax protein (Chen and Chuang, 1999), but activates the survival-promoting protein kinase, Akt, (Chalecka-Franaszek and Chuang, 1999) in cerebellar granule cells.

In addition to protection against β-amyloid peptide-induced neurotoxicity shown in the present study, lithium has also been reported to reduce hyperphosphorylation of tau, a microtubule-binding protein that forms paired helical filaments in neurons of the brains of patients with Alzheimer's disease (Hong et al., 1997; Muñoz-Montaño et al., 1997). Since our paper was submitted, Alvarez et al. (1999) reported that lithium, at a relatively high concentration, reduces β-amyloid peptide-(1–40)-induced hyperphosphorylation of tau protein and cell death of rat cortical neurons. Their observations and our results suggest that lithium may be used to control the progression of neurodegeneration in the brain of Alzheimer's disease. In this context, it is interesting to note that lithium has been used to treat Alzheimer's disease with mixed results. Some studies show promising beneficial outcomes, while others indicate little or no improvement (for review, see Sky and Grossberg, 1994). In light of the neuroprotective effects of lithium and its ability to induce Bcl-2, the use of this drug to treat Alzheimer's disease should be reevaluated and reexamined, particularly for those patients at the early stage of this neurodegenerative disease.

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