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Survival-Promoting Mechanism via PI3-Kinase in Cerebellar Granule Neurons

KOJI SHIMOKE

Division of Protein Biosynthesis,

Institute for Protein Research,

Osaka University.

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ABBREVIATIONS

BDNF	Brain-derived neurotrophic factor
EGF	Epidermal growth factor
Gab1	Grb2-associated binder 1
HS	Horse serum
IGF-1	Insulin-like growth factor-1
IRS-1/2	Insulin receptor substrate-1/2
JNK	c-Jun N-terminal kinase
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide
NGF	Nerve growth factor
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
PI3-K	Phosphatidylinositol 3-kinase
PH	Pleckstrin homology
PNCS	Precolostrum newborn calf serum
PLC-γ	Phospholipase C-γ 1
SH2	Src homology 2
SDS	Sodium dodecyl sulfate

SUMMARY

Most of the higher organisms, such as vertebrates, have their bodies controlled by the nervous system. In the nervous system, neurons have key roles in the neuronal functions. Most of neurons never proliferate after the neuronal network has been constructed in a mature form. Thus, the mature nervous system must be established in the process of development. There is a hypothetical theory for construction of the nervous system; that is, trophic factors select the essential population of neurons. During early development, excess amounts of neurons are produced and about a half of them are eliminated. In the process to construct the neuronal network, the target cells release neurotrophic factors which can promote the neurite outgrowth and prevent the neuronal death. As the released amounts of neurotrophic factors are not enough for all neurons to survive, only the neuron which is able to receive sufficient neurotrophic factors survive and then work to construct the mature neuronal network. This selection system among neurons is thought to be a way to construct the exact and physiologically relevant neuronal network. Therefore, the mature nervous system is established as the result of cell death of about a half neurons produced.

The cell death, also termed as apoptosis, occurs not only in the neurons

under the developmental process but also in the mature neurons by exogenous toxic stimuli. The cell death might cause a serious effect on the mature nervous system. Interestingly, this cell death in the mature neurons can be prevented by neurotrophin, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Membrane depolarization by a high concentration of potassium (HK^+) also prevents the cell death. Therefore, the importance of the study about the actions of the neurotrophins and HK^+ is increasing at present.

In this issue, I describe the intracellular mechanism of the BDNF- and HK^+-mediated prevention of cell death in cultured cerebellar granule neurons. In part 1, I would like to address to examine which molecules are involved in the BDNF-and HK^+-initiated cell survival signal. For such purpose, I investigated the effects of specific inhibitors of mitogen-activated protein kinase (MAPK) kinase, phospholipase C-γ 1 (PLC-γ) and phosphatidylinositol 3-kinase (PI3-kinase). PD98059 and U-73122, inhibitors of MAPK kinase and PLC-γ, respectively, did not affect the cell viability. On the other hand, LY294002 and wortmannin, both of which are specific inhibitors of PI3-kinase, induced the cell death. To analyze further, I measured the activity of PI3-kinase of cells in the condition of HK^+ medium or low potassium (LK^+) medium containing BDNF in the absence or presence of wortmannin. I observed that wortmannin decreased the PI3-kinase activity. These results indicate that PI3-kinase is an obligatory mediator of the cell

survival signal in cerebellar granule neurons.

In part 2, I examined the possibility that Akt can contribute to the survival pathway which is initiated by BDNF or HK^+. The activity of Akt was measured in the cells in the culture condition of LK^+ with BDNF, or in the culture condition of HK^+ with specific inhibitor of PI3-K, LY294002 or wortmannin. I observed that the both activities of PI3-K and Akt decreased when either inhibitor was added. A synthetic phospholipid derivative, dipalmytoyl-3,4-bisphosphate, which potentially activates Akt, prevented apoptosis occurred in the culture condition of LK^+. I also observed an increase of the Akt activity in the presence of this synthetic lipid. On the other hand, rapamycin, which suppresses the activation of $p70^{S6K}$, had no effect on the cell viability. These results indicate that two different survival-promoting stimuli, BDNF and HK^+, can prevent apoptosis of cerebellar granule neurons via the same PI3-K-Akt signaling pathway but not via $p70^{S6K}$. In addition, I also analyzed the signaling pathway downstream of Akt. LY294002, a specific inhibitor of PI3-K, increased the c-Jun N-terminal kinase (JNK) activity and the c-Jun expression in the following three different conditions of HK^+, LK^++BDNF and LK^++IGF-1. These results indicate that the PI3-K - Akt/PKB pathway suppresses the activation of JNK and the c-Jun expression, and prevents the neuronal cell death in cerebellar granule neurons.

Part 1:

Role of PI3-Kinase in Prevention of Low K^+-Induced Apoptosis of Cerebellar Granule Neurons

Abstract

Brain derived neurotrophic factor (BDNF), high potassium (HK^+) medium and insulin like growth factor-1 (IGF-1) prevent apoptosis of cerebellar granule neurons. To examine which molecules are involved in the BDNF- and HK^+-initiated cell survival signal, I examined the effects of specific inhibitors of MAPK kinase, PLC-γ and PI3-kinase. PD98059 and U-73122, inhibitors of MAPK kinase and PLC-γ, respectively, did not affect the cell viability. On the other hand, LY294002 and wortmannin, both of which are specific inhibitors of PI3-kinase, induced the cell death that were estimated by the MTT assay, Hoechst staining or the TUNEL method. To analyze further, I measured the activity of PI3-kinase of cells in the condition of HK^+ medium or low potassium (LK^+) medium with BDNF in the absence or presence of wortmannin. I observed that wortmannin decreased the PI3-kinase activity. These results indicate that PI3-kinase is an obligatory mediator of the cell survival signal in cerebellar granule neurons. In addition, an immunoprecipitaton analysis using anti-p85 subunit of PI3-kinase antibody suggested that this activity of PI3-kinase is activated by binding to a tyrosine-phosphorylated 95-kD protein, which might be Grb2 associated binder 1 (Gab1).

Key words:

apoptosis, wortmannin, LY294002, brain-derived neurotrophic factor (BDNF), high potassium, phosphatidylinositol 3-kinase (PI3-K), Ca^{2+} influx, TrkB

1. Introduction

Activation of receptor tyrosine kinases by their ligands causes several molecular events in intact cells. Autophosphorylation of Trk family receptors (Trks) initiates the binding of some signaling molecules to the dimerized receptor and thus localizes these molecules to cell membrane (Jing et al., 1992). This localization is achieved by the phosphotyrosine-binding (PTB) domain or the src homology 2 (SH2) domain (McGlade et al., 1992; Michael and Williams, 1994; Kavanaugh et al., 1995). These interactions are highly site-specific and each binding molecule has a crucial role in its signaling pathway. Trks can interact with several PTB- or SH2-domain containing signaling molecules such as Shc (Schlessinger, 1993), phospholipase C-γ 1 (PLC-γ) (Kim et al., 1991; Obermeier et al. and Middlemas et al., 1994) and insulin receptor substrate-1/2 (IRS-1/2) (Yamada et al., 1997). These molecules are phosphorylated immediately after their binding to Trks (Schlessinger and Ullich., 1992). And then, Shc activates the Ras signaling pathway leading to the activation of MAPK (Rozakis-Adock et al., 1992; Nishida and Gotoh, 1933; Egan et al., 1993). This pathway manifests the neuronal differentiation (Guerrero et al., 1988; Wood et al., 1993; Cowley et al., 1994) by neurotrophins including BDNF, and may be redundant with the PLC-γ pathway (Stephens et al., 1994).

IRS-1 and -2 are the adaptor proteins which are phosphorylated by the insulin receptor after induction with insulin (Backer et al., 1992; Holgado-Madruga et al., 1996; Yenush and White, 1997). Recently, Yamada et al. (1997) revealed that IRS-1/2 are directly associated with phosphatidylinositol-3 kinase (PI3-K) to promote the signal transduction by BDNF in cortical neurons, indicating that Trks initiate the PI3-K pathway through IRS-1/2. The responses of cells in which PI3-K is activated by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, insulin-like growth factors or nerve growth factor (NGF) are well understood (Ruderman et al., 1990; Hu, P. et al., 1992; Carter and Downes, 1992; Kimura et al., 1994). For instance, membrane ruffling (Kotani et al., 1994), glucose metabolism (Okada et al:, 1994), actin regeneration (Wymann and Arcaro, 1994) and inhibition of cell death (D`Mello et al, 1997; Miller et al, 1997; Philpott et al, 1997; Shimoke et al, 1997; Yao and Cooper, 1997) are the typical examples of various PI3-K-mediated actions. However, the physiological functions of PI3-K are not fully understood. This enzyme was first discovered in anti-v-Src immunoprecipitates and was found to have the ability of phosphorylation of the D-3 position of phosphatidylinositol ring (Sugimoto et al., 1984; Auger et al., 1989). PI3-K consists of two subunits, p110 catalytic subunit and p85 regulatory subunit which contains two SH2 domains and one SH3 domain (Carpenter and Shoelson, 1993). The interaction between the SH2 domain of p85 subunit and the phosphotyrosine-containing

binding motif is necessary for the PI3-K activation (Koch et al., 1991; Kaplan and Stephens, 1994). In analysis of PI3-K itself, potent and specific inhibitors, LY294002 and wortmannin, were developed. LY294002 is a reversible inhibitor, and wortmannin is an irreversible one that bind covalently to the active site of this enzyme (Vlahos et al., 1994; Wymann et al., 1996). Great progress about functions of PI3-K has been made using these tools.

This enzyme produces three types of phospholipid, that is, phosphatidylinositol-3-monophosphate (PI-3-P), phosphatidylinositol-3,4-bisphosphate (PI-3,4-P_2) and phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3) (Cantley et al., 1991). The amounts of PI-3,4-P_2 and PI-3,4,5-P_3, but not PI-3-P, increases transiently in the cells after stimulation by cytokines or trophic factors (Auger et al., 1989). In addition, some reports revealed that PKC isoforms (δ , ε , η) (Toker et al., 1994) and the serine / threonine kinase Akt, also termed as PKB, were activated by PI-3,4-P_2 and/or PI-3,4,5-P_3, as expected from the changes in lipid amounts. Recently, it has been reported that the PI3-K pathway leads to the phosphorylation of BAD, and could prevent the BAX activation and the neuronal cell death.

In this part, I show that BDNF and high potassium (HK^+) medium have the survival-promoting effect in cultured cerebellar granule neurons and that this effect is mediated by PI3-K, but not by MAP kinase or PLC-γ .

2. Materials and Methods

2.1. Cerebellar granule neuron cultures

Primary cultures of cerebellar granule neurons from dissociated cerebella of 9-day-old rats were prepared as reported (Hatanaka et al., 1988; Levi et al., 1984; Kubo et al., 1995). Briefly, cells were gently dissociated with a plastic pipette after digestion with papain (90 U/ml, Worthington) at 37℃. The cells were then seeded onto 48-well plates (0.65 cm^2/well; Sumitomo Bakelite) coated with polyethyleneimine in culture medium consists of 5% (v/v) precolostrum newborn calf serum, 5% (v/v) heat-inactivated horse serum, 1% (v/v) rat serum and 89% 1:1 mixture of Dulbecco's modified Eagle's (DME) medium and Ham's F12 medium containing 15 mM HEPES buffer, pH 7.4, 30 nM selenium and 1.9 mg/ml sodium bicarbonate. The cells were seeded at a density of 5×10^5 cells/cm^2. For Hoechst 33,258 staining, cells were seeded onto glass coverslips coated with polyethyleneimine. After one day of culture in a humidified CO_2 incubator, the medium was changed to high K^+ minimum essential medium (MEM) supplemented with 5% (v/v) heat-inactivated horse serum and 1 μM cytosine arabinoside. MEM was supplemented with 2.2 mg/ml glucose, 0.5 mg/ml sodium bicarbonate and 0.94 mg/ml sodium chloride. High K^+ MEM was

prepared by increasing the $KHCO_3$ and KCl concentration from the normal value 5.4 mM to 26 mM, with the omission of the corresponding concentration of $NaHCO_3$. After 4 days in culture, the medium was changed to serum-free MEM or serum-free high K^+ MEM with or without the factors as indicated. When required, wortmannin (100 nM), LY294002 (10 μM), PD98059, U-73122 or rapamycin (these are added at an indicated concentration) was added.

2.2. Assay of neuronal survival

Neuronal survival was determined by the MTT assay (Kubo et al., 1995). A modification (Hansen et al., 1989) of the original procedure (Mosmann, 1983) was used to measure the mitochondrial function. Briefly, the tetrazolium salt MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide) was added to culture (1 mg/ml at a final concentration). After a 2 hr incubation at 37℃, the reaction was stopped by adding lysing buffer (20% SDS in 50 % aqueous N,N-dimethyl formamide solution, pH 4.7). The absorbance was measured spectrophotometrically at 570 nm after a further overnight incubation at 37℃. The percent survival was defined as [Abs.(experimental - blank)/Abs.(control - blank)] $\times$100, and the blank was the value taken from wells without cells.

2.3. Hoechst 33258 staining

After 24 hours of exposure to LY294002 or wortmannin, granule neurons were fixed in 4% paraformaldehyde for 20 min, rinsed twice with phosphate-buffered saline (PBS), stained with 1 μ g/ml Hoechst 33258 (Wako) in PBS for 15 min, and then examined under UV illumination using a Nikon fluorescence microscope.

2.4. Immunocytochemistry

For terminal deoxynucleotide transferase-mediated dUTP fluorescein nick end-labeling (TUNEL) staining, the cultured cells were fixed with 4 % paraformaldehyde for 20 min and labeled using a biotin 16 dUTP (Boehringer Mannheim) as a substrate of the transferase. Cells were then stained using the Vectastain ABC kit (Vector Lab.), 0.02 % (w/v) 3,3'-diaminobenzidine 4-HCl (DAB) and 0.1 % (w/v) $(NH_4)_2Ni(SO_4)_2$.

For MAP2 staining, cells were incubated with anti-MAP2 antibody (1:5000) in buffer G` (0.3 % Triton X-100, 5 % normal goat serum, 0.01 % NaN_3) overnight at 4 ℃ then stained using the Vectastain ABC kit (Vector Lab.), 0.02 % (w/v) 3,3'-diaminobenzidine 4-HCl (DAB) and 0.3 % H_2O_2.

2.5. Immunoblotting

PI3-kinase was immunoprecipitated with anti-p85 subunit of PI3-K antibody in lysis buffer containing 1 % Triton X-100, 20 mM Tris-HCl (pH, 7.6), 137 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml aprotinin and 20 mM NaF. The insoluble debris was removed from lysates by a brief centrifugation and the protein concentration of clarified lysates was normalized by the BCA protein assay. The anti-p85 immonoprecipitates were washed three times and electrophoresed on SDS-PAGE and then transferred onto nitrocellose membranes. The membranes were blotted with anti-phosphotyrosine antibody (4G10) and the secondary antibody, horse-radish peroxidase-conjugated anti-mouse IgG antibody. The antibody-bounded proteins were detected by the enhanced chemiluminescence system (Amersham).

2.6. Neurotrophin

BDNF, produced in *E. coli* and purified as described, was provided by Amgen-Regeneron Partners. BDNF was dissolved at 25 μ g/ml in PBS containing 1 mg/ml BSA as a stock solution and added to the cultures at 50 ng/ml, which concentration is maximum dose for the survival of cerebellar granule neurons.

2.7. In vitro kinase assay

The PI3 kinase activity was measured as described method (Hu and Soltoff, 1992; Rameh, 1995). Cerebellar granule neurons were cultured for 4 days, then medium was replaced with serum-free culture medium. After 1 day, cells were washed twice with buffer A (137 mM NaCl, 20 mM Tris-HCl (pH,7.5), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM sodium orthovanadate). Then, cells were lysed with buffer B (10 % glycerol, 1 % Nonidet P-40, 1 mM PMSF dissolved in buffer A) and centrifuged at 15,000×g for 30 minutes. Aliquots were incubated with anti-phosphotyrosine antibody (PY20) on ice for 3 hours after the BCA protein assay, and precipitated with protein A-Sepharose beads for 2 hours. Then, *in vitro* kinase reaction was carried out with protein A-Sepharose beads and phosphatidylinositol as the substrate in 70 μl of reaction buffer (100 mM NaCl, 20 mM Tris-HCl, 0.5 mM EGTA) in the presence of [γ-^{32}P] ATP (10 μCi/sample) at 25 ℃ for 7 minutes. The reaction was stopped by addition of a solution of methanol, chloroform and 10 M HCl (50:50:1, by volume). The lower phase was removed and extracted again with chloroform. The chloroform extract was spotted on thin-layer chromatography (TLC) plates pretreated with potassium oxalate, and the TLC plates were then developed with chlroform:acetone:methanol:acetic acid:water (40:15:13:12:7, by volume), and radioactivity was visualized by autoradiography. The TLC plates were exposed to Kodak diagnostic films.

3. Results

3.1. Specific inhibitors of PI3-K, LY294002 and wortmannin, block the survival effects of BDNF and HK^+.

When cerebellar granule neurons are cultured in serum-free medium containing the normal concentration of potassium (5 mM), apoptosis occurs (D`Mello et al., 1993; Kubo et al., 1995). This can be prevented by several factors, including high $K^+$, BDNF and IGF-1 (D`Mello et al., 1993; Kubo et al., 1995). To examine the survival pathway induced by high K^+ or BDNF, I used LY294002 and wortmannin, specific inhibitors of PI3-K (Okada at al., 1992a, 1992b; Kimura et al., 1994) As shown in Fig. 1, these inhibitors effectively blocked the BDNF-enhanced survival of cerebellar granule neurons. The survival-promoting effect of high K^+ was also prevented by both LY294002 and wortmannin. These results indicate that the activity of PI-3 kinase is required for both the BDNF- and high K^+-mediated survival promotion of cerebellar granule neurons.

3.2. Prevention of the PI3-kinase activity induces apoptosis in cerebellar granule neurons.

To characterize the LY294002- or wortmannin-induced death of cerebellar granule neurons cultured with BDNF or high K^+, I stained the nuclei of cells with Hoechst 33258. The chromatin of granule neurons cultured with BDNF or high K^+ was widely dispersed (Fig. 2A and C). When LY294002 was added to the culture medium of either condition, the chromatin of cells condensed and was brightly stained (Fig. 2B and D). Furthermore, DNA fragmentation was also detected by the TUNEL method. As shown in Fig. 3B and D, cerebellar granule neurons cultured with BDNF and high K^+ in the presence of LY294002, were stained positively by the TUNEL method in contrast to those cultured in the absence of the inhibitor (Fig. 3A and C). These results indicate that cerebellar granule neurons cultured with BDNF or high K^+ die in an apoptotic manner in the presence of LY294002. A similar effect was observed with another inhibitor, wortmannin (data not shown).

In order to characterize the wortmannin- and LY294002-induced apoptosis of granule neurons furthermore, I examined the effects of cycloheximide and actinomycin D, which are protein and RNA synthesis inhibitors, respectively. Both inhibitors significantly prevented the wortmannin- and LY294002-induced apoptosis (Fig. 4). These results indicate that *de novo* macromolecule synthesis is required for apoptosis elicited by the presence of these inhibitors.

3.3. Wortmannin decreases the PI3-K activity in cerebellar granule neurons.

I measured the activity of PI3-K in cerebellar granule neurons 1 day after changing the medium to the condition of high K^+, low K^+ or low K^+ plus BDNF. As shown in Fig. 5, the PI3-K activity of cerebellar granule neurons in high K^+ medium was higher than that in low K^+ medium. The PI3-K activity in low K^+ medium containing 50 ng/ml BDNF was increased in comparison with that in low K^+ medium without BDNF. These results indicate that the treatment with BDNF increased the activity of PI-3 kinase, while the treatment with low K^+ medium decreased the activity of PI3-K in cerebellar granule neurons.

Then, I measured the PI3-K activity in cerebellar granule neurons in the presence of wortmannin, a specific inhibitor of this enzyme. Wortmannin decreased the PI3-K activity in the cells cultured with high K^+ medium as shown in Fig. 5. A similar decrease in the PI3-K activity by wortmannin was also observed in cells cultured with low K^+ medium containing BDNF. These results strongly suggest that PI3-K is involved in the signaling pathway leading to the survival of cerebellar granule neurons.

3.4. MAP Kinase and PLC-γ are not involved in the survival mechanism.

The Trk receptor tyrosine kinase, directly phosphorylates Shc and PLC-γ. Shc leads to the activation of the Ras-MAP kinase (MAPK) pathway, and PLC-γ leads to the activation of Raf-1 as a redundant signal with the

Ras-MAPK pathway. Thus, the activation of Raf-1 by PLC-γ could crosstalk to the Ras-MAPK pathway. In rat sympathetic neurons, Ras, but not MAPK, is an obligatory mediator to suppress the cell death. This means the existence of a bifurcation in the downstream pathway of Ras. Therefore, I examined the possible roles of the MAPK pathway and the PLC-γ pathway in suppression of apoptosis in cerebellar granule neurons, by using inhibitors of MAPK kinase and PLC-γ, PD98059 and U-73122, respectively. As shown in Fig. 6, PD98059 did not suppress the cell viability at any concentrations which was able to reduce the MAPK activity. Similarly, U-73122 did not show the significant decreases in the viability of cerebellar granule neurons. These results indicate that MAP kinase and PLC-γ are not involved in the BDNF- and HK^+-mediated survival-promoting pathway.

3.5. HK^+ and BDNF activate PI3-K through an unknown p95 protein.

Trophic factors and cytokines bind to their receptors on the cell surface and activate several intracellular molecules. BDNF and IGF-1 can activate TrkB and the IGF-1 receptor, respectively, on cerebellar granule neurons. As shown in Fig. 5, I observed the PI3-K activity even 1 day after the HK^+ or BDNF stimulation. To identify the tyrosine-phsphorylated proteins bound to PI3-kinase 5 minutes or 1 day after these stimulations, immunoprecipitation with anti-p85 antibody was carried out. As shown in Fig. 7, a 95-kD or a

180-kD tyrosine-phosphorylated protein was detected when cells were treated with BDNF or IGF-1 for 5 minutes. The phosphorylation of 95-kD protein was decreased 1 day after the addition of BDNF. The same 95-kD band was observed 1 day after changing medium to HK^+. However, the phosphorylation of 95-kD protein was not detected 5 minutes after changing medium to HK^+. Thus, this protein is a candidate which binds to PI3-K and activates the PI3-K activity in the signaling pathway. The 95-kD tyrosine-phosphorylated protein may be Gab1, which is known to bind to PI3-K in response to NGF in PC12 cells (Holgado-Madruga., 1997).

Taken together, I show that PI3-K which is activated by BDNF or HK^+ medium mediates the survival-promoting pathway in cerebellar granule neurons (Fig. 8).

4. Discussion

In the development of neurons, excess number of neurons are produced and about a half of them die (Oppenheim., 1991). The time of the cell death (apoptosis) is limited to the period of the formation of synapses with the target cells, and the neurons which fail to obtain sufficient amounts of trophic factor(s) released from the target cells are eliminated. This selection system among neurons is considered to be a way to form the exact and physiologically relevant neuronal network to fulfill the neuronal functions. Mature neurons which correctly execute their functions, however, undergo apoptosis by exogenous toxic stimuli. These phenomena may cause the neuronal degenerative diseases and then lead to the disintegration of individuals. The mechanism underlying the cell death has been analyzed using an *in vitro* model system. I use cultured rat cerebellar granule neurons because of their abundance and their homogeneity. The low concentration of potassium (LK^+) in the medium induces apoptosis of cerebellar granule neurons and this apoptosis is prevented by the high concentration of potassium (HK^+), BDNF, IGF-1, bFGF or cAMP (Fernandez-Sanchez et al., D'Mello, 1993; Kubo et al., 1995). BDNF is one of the members of neurotrophins, which also contain NGF, NT-4/5 and NT-3. Neurotrophins induce their functions, including the prevention of apoptosis by binding to their receptors, Trks, and

the Trk receptors activate several intracellular signaling molecules by interacting with these molecules, directly or indirectly via docking proteins. Shc and PLC-γ bind to Trks directly, then Shc activates the Ras-MAP kinase pathway, and PLC-γ activates PKC via the production of inositol triphosphate and diacylglycerol (Nishizuka, 1988; Vetter et al., 1991; Berridge, 1993). The Ras-MAP kinase signaling pathway leads to the neuronal differentiation and the PLC-γ pathway is considered to be redundant with the Ras-MAP kinase pathway in PC12 cells (Stephens et al., 1994). Some investigators showed that MAP kinase is not essential component of the survival-promoting effect in sympathetic neurons (Virdee and Tolkovsky., 1995, 1996) and in cerebellar granule neurons (Gunn-Moor et al., 1997). I observed that the PLC-γ pathway was not important for the survival-promoting effect, either. This result suggests that the calcium-sensitive PKC which can be activated by PLC-γ is not involved in the survival-promoting pathway.

One of the lipid-modifying kinases, PI3-K, is also activated by trophic factors including neurotrophins (Ohmichi et al., 1992; Soltoff et al., 1992; Backer et al. 1992; Carter et al., 1992; Okada et al., 1994; Nonomura et al., 1996). Recent reports showed that the serum deprivation-induced apoptosis was prevented by PI3-K in PC12 cells (Yao et al., 1995), and in oligodendrocytes and fibroblasts (Vemuri et al., 1996; Yao et al., 1996). On the other hand, LK^+-induced apoptosis was prevented by PI3-K which was activated by IGF-1, in cerebellar granule neurons (Dudeck, et al., 1997; D`Mello, et al., 1997;

Miller, et al., 1997; Shimoke, 1997). In this part, by using specific inhibitors of PI3-K, LY294002 and wortmannin (Okada et al., 1994; Vlahos et al., 1994), I also show that BDNF and HK^+ promote the cell survival via PI3-K. It was reported that LY294002 is a reversible inhibitor which competes with ATP for binding to PI3-K (Vlashos et al., 1994; Rameh et al., 1995), but wortmannin is an irreversible one by covalent binding to the active site of PI3-K (Wymann et al., 1996). Both of these inhibitors are a highly specific to PI3-K. I observed the inhibitory effect of them on the cell survival of cerebellar granule neurons. I also measured the PI3-K activity in the presence of wortmannin in the conditions of HK^+ and LK^++ BDNF, and found that wortmannin decreased the PI3-K activity. However, there was not a parallel correlation between the decreased percentage of the viability and that of the PI3-K activity. Both the viability and the PI3-K activity were measured 24 hours after the medium change to HK^+ or LK^++ BDNF. Thus, the absence of correlation may be due to the time lag from the effective point of the PI3-K activity. To confirm my results, the dominant negative form of PI3-K should be used in cerebellar granule neurons.

A recent report about the mechanism of the PI3-K activation showed that its activation initiated by BDNF was mediated by IRS-1/2 in cortical neurons (Yamada et al., 1997). In addition, Holgado-Madruga et al. (1997) showed that the PI3-K activation was mediated by Grb2-associated binder-1 (Gab1) in PC12 cells. My result show that there is a tyrosine-phosphorylated 95-kD protein bound to PI3-K, which may be identical to Gab1. Therefore, the PI3-K

activation may be mediated by Gab1 in cerebellar granule neurons. But I could not detect IRS-1/2 in the anti-PI3-K immunoprecipitate of cerebellar granule neurons treated wuth BDNF. This might be due to the difference in cell-types. These results mean that PI3-K is not activated by binding directly to Trk receptor, in contrast to the previous reports (Soltoff et al., 1992; Obermeier., 1993). I also observed the PI3-K activation in the condition of HK^+ medium. It is suggested that the activation of PI3-K by HK^+ is mediated by the calcium influx through the voltage-activated calcium channel (Gallo et al., 1987). The activation of PI3-K induced by the calcium influx may occur through two possible mechanisms. First, the influxed calcium may directly activate the intracellular kinases (Rosen et al., 1994; Rusanescu et al., 1995; Finkbeiner et al., 1996) which may activate PI3-K. For instance, the calcium-dependent kinase, PYK2, and a calcium bound-calmodulin may activate PI3-K directry, or PYK2 may activate PI3-K indirectly though Src or Ras (Lev et al., 1995; Rusanescu et al., 1995). Second, the influxed calcium may initiate the production of trophic factors and these factors may activate the intracellular signaling through an autocrine loop (Ghosh et al., 1994; Sano et al., 1996; Ono et al., 1997). I observed that HK^+ and BDNF had significant additive effect and the HK^+-induced survival-promoting effect was not inhibited by the anti-BDNF antibody (Shimoke et al., 1997). These data suggest that BDNF is not involved in the survival-promotion by HK^+. Some parts of the survival-promoting effect by HK^+ may depend on the parathyroid

hormone-related protein which is produced by HK^+ (Ono et al., 1997).

Dudek et al. and D`Mello et al. (1997) showed that LK^+-induced cell death was prevented by IGF-1 and HK^+, and that LY294002 and wortmannin inhibited the IGF-1-initiated- but not the HK^+-initiated survival promotion. The discrepancy between my and their results about the effect of these inhibitors may be due to some differences in culture conditions. In addition, Courtney et al. (1997) showed HK^+ was effective on the cell survival of cerebellar granule neurons after 6 days in *in vitro* culture. At present, the mechanism of responsiveness to HK^+ after 6 days in their conditions is not clear.

The results presented here suggest that BDNF and HK^+ require the PI3-K activity for their survival-promoting effects, although the molecular mechanisms of these effects may be different. Cerebellar granule neurons are a useful system to investigate the molecular mechanisms of these survival-promoting effects because of their homogeneity. By using this system, some investigators have already studied the signaling pathway downstream of PI3-K and have observed that the phosphorylation of BAD via Akt (Dudek et al., 1997; Franke et al., 1997; Datta et al., 1997; Peso et al., 1997) and the activation of calcium-insensitive isoform of PKC via the products of PI3-K, PI-3,4-P_2 and PI-3,4,5-P_3 (Toker et al., 1994; Zirrgiebel et al., 1995; Courtney et al., 1997). Therefore, in part 2, I also investigate the survival-promoting pathway downstream of PI3-K in cerebellar granule neurons.

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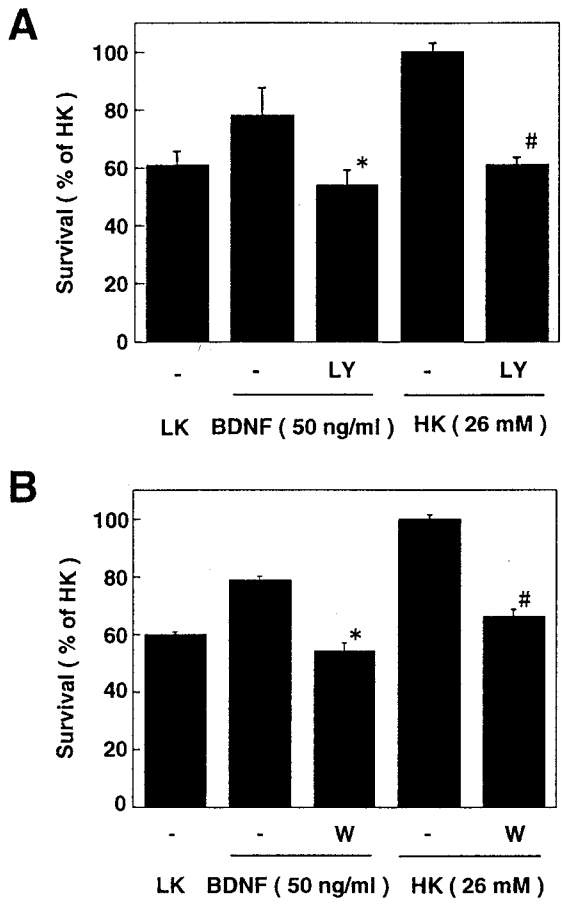


Figure 1. Inhibition of the survival-promoting effects of BDNF and high K^+ on cultured cerebellar granule neurons by LY294002 and wortmannin. The medium was changed to serum-free MEM in the absence (LK) or presence of BDNF (50 ng/ml) (BDNF, BDNF+LY, BDNF+W) or serum-free high K^+ (26 mM) MEM (HK, HK+LY, HK+W), then 10 μM LY294002 (LY) or 100 nM wortmannin (W) (every 5 hours) was added to the cultures (BDNF+LY, BDNF+W, HK+LY or HK+W). After 24 hours, the neuronal survival was determined by the MTT assay as described in **Materials and Methods**. The mean value obtained from cells maintained in serum-free high K^+ MEM (HK) without inhibitors was defined as the control. Values are means ± S.E.M. (n=4). Statistical analysis was performed by Student's t test. $^*P<0.001$, vs. BDNF and $^\#P<0.001$, vs. HK.

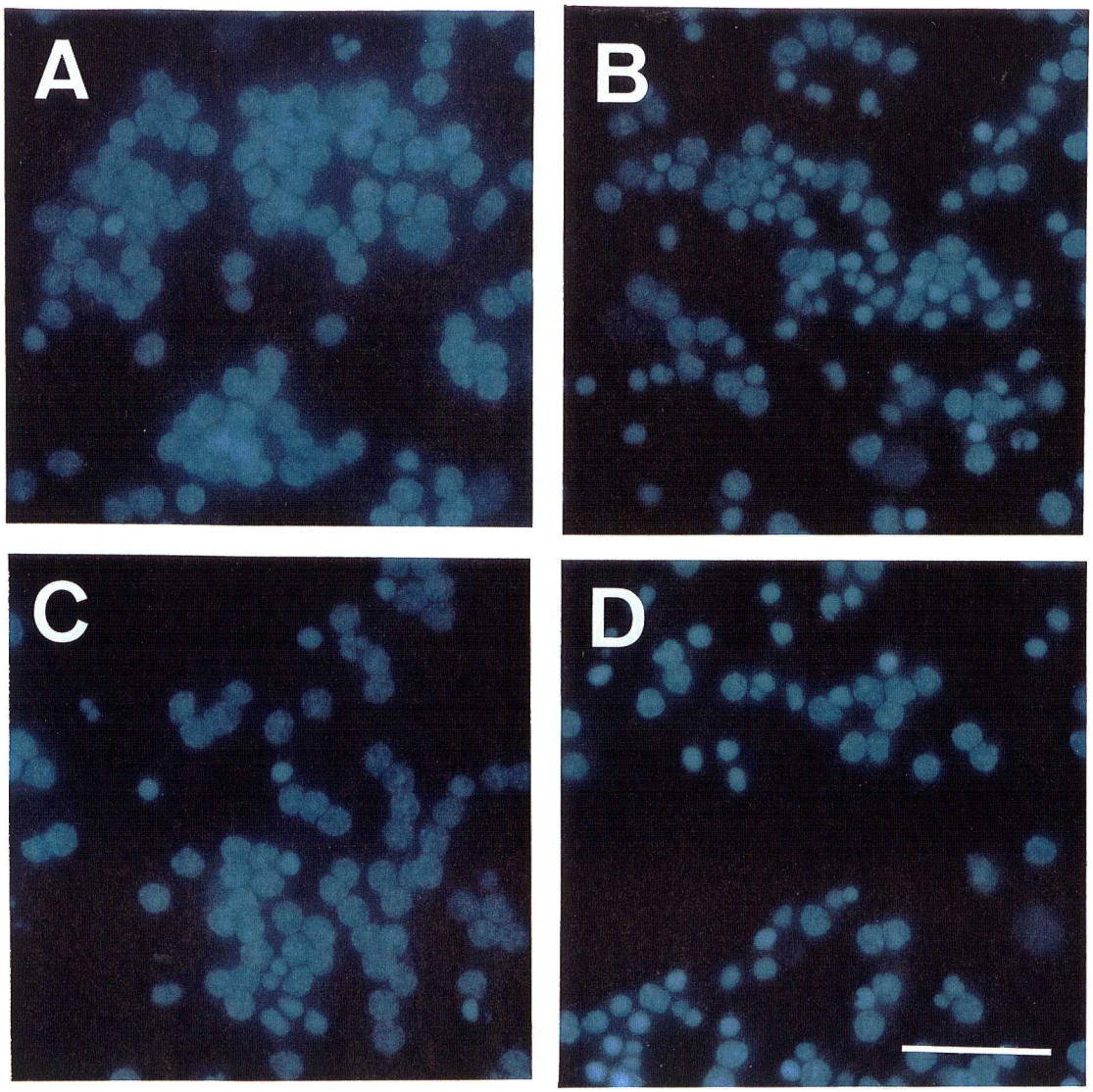


Figure 2. Morphological features of the nuclei of cultured cerebellar granule neurons exposed to LY294002. After 24 hours in serum-free high K^+ MEM in the absence (A) or presence (B) of 10 μM LY294002, or in serum-free MEM containing 50 ng/ml BDNF with (D) or without (C) 10 μM LY294002, the nuclei of cells were stained with Hoechst 33258 as described in **Materials and Methods**. Photographs were taken using a fluorescent microscopy. Scale bar represents 40 μm.

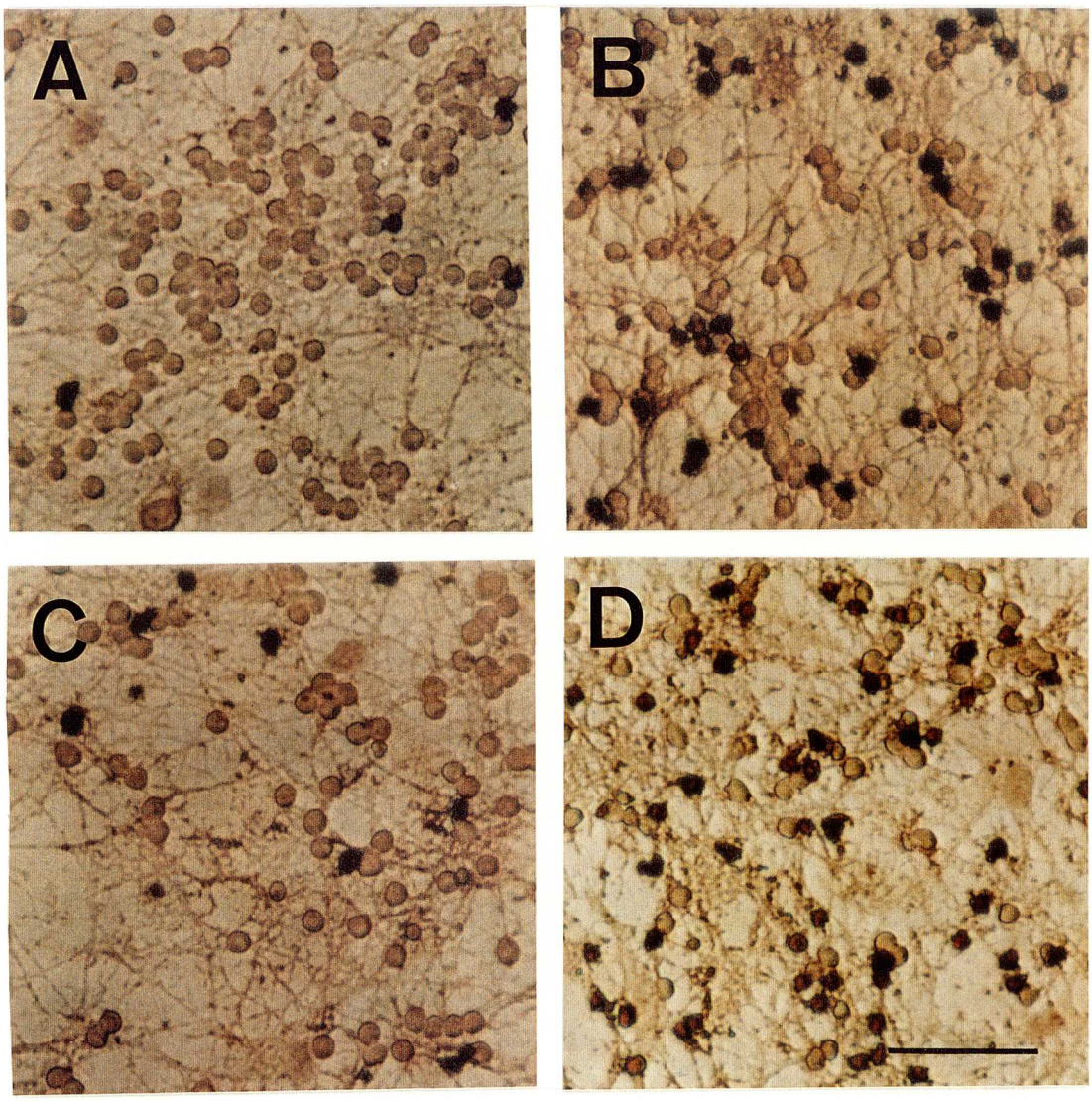


Figure 3. DNA fragmentation occurred after the addition of LY294002. Cultured cerebellar granule neurons were cultured under serum-free conditions as described in Figure 2, and DNA fragmentation was detected by the TUNEL method as descrived in **Materials and Methods**. A, High K^+ MEM without LY294002; B, high K^+ MEM with 10 μM LY294002; C, low K^+ MEM containing 50 ng/ml BDNF without LY294002; D, low K^+ MEM containing BDNF with 10 μM LY294002. Photographs were taken using a bright-field microscopy. Scale bar represents 45 μm..

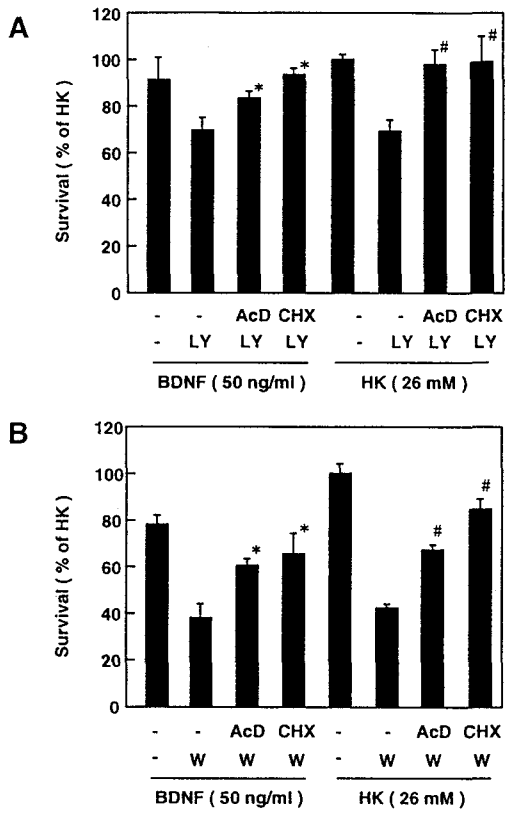
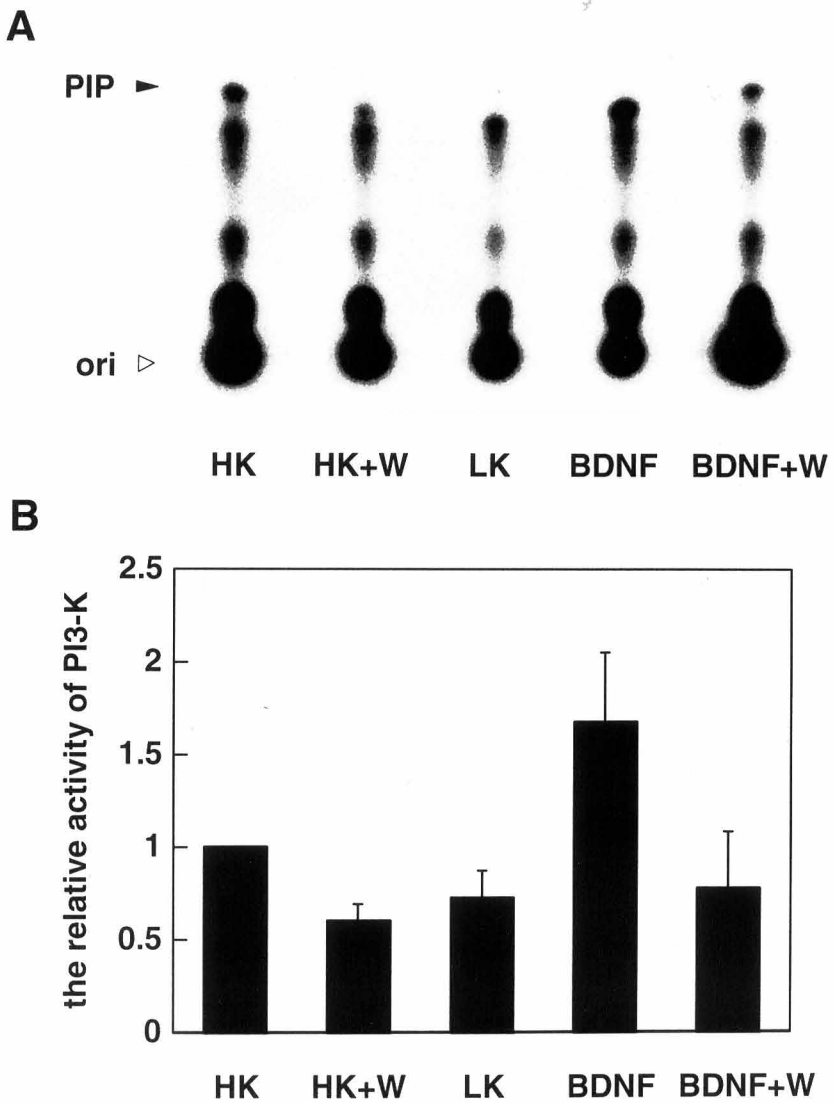


Figure 4. Effects of RNA and protein synthesis inhibitors on the cell death of cerebellar granule neurons exposed to LY294002 and wortmannin. The medium was shifted to serum-free high K^+ (26 mM) MEM (HK) or serum-free MEM containing 50 ng/ml BDNF (BDNF), without (-) or with 10 μM LY294002 (LY), 100 nM wortmannin (W), LY294002 plus 0.1 μM actinomycin D (LY+AcD), wortmannin plus 0.1 μM actinomycin D (W+AcD), LY294002 plus 0.3 μM cycloheximide (LY+CHX) or wortmannin plus 0.3 μM cycloheximide (W+CHX). After 24 hours, the neuronal survival was estimated by the MTT assay as described in **Materials and Methods**. The mean value obtained from cells maintained in serum-free high K^+ MEM (HK) without additives was used as the control. Values are means ± S.E.M. (n=4). Statistical analysis was performed by ANOVA. A, $^*P<0.001$, vs. BDNF+W. $^\#P<0.01$, vs. HK+W. B, $^*P<0.01$, vs. BDNF+LY. $^\#P<0.01$, vs..HK+LY.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the relative activity of PI3-K
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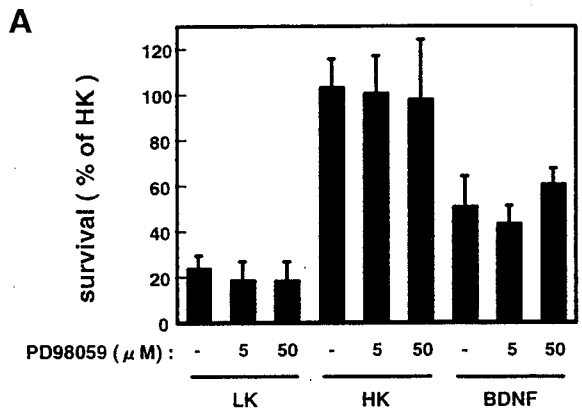
LK
BDNF
BDNF+W

Figure 5. Decreased activity of PI3-kinase by wortmannin.
Cultured cerebellar granule neurons were maintained in high K^+ MEM containing 5 % heat-inactivated horse serum and 1 μM cytosine arabinoside for 4 days. Then, cells were subjected to serum-free high K^+ MEM (HK, HK+W) or serum-free MEM without (LK) or with 50 ng/ml BDNF (B, B+W) for 1 day. The activity of PI3-kinase was measured 30 minutes after the addition of 100 nM wortmannin (HK+W, B+W) as described in **Materials and Methods**. The open arrowhead indicates an origin. The filled arrowhead indicates the position of phosphatidylinositol 3-monophosphate (PI-3-P), the product of PI3-kinase, estimated from the position of phosphatidylinositol 4-monophosphate as a standard. The radioactivity of TLC plates was visualized by autoradiography. The TLC plates were exposed to Kodak diagnostic films. The densitometry was performed using BAS2000 (Fuji Film). Relative intensities (means$\pm$ S.E.M., n=3) were as follows. HK: 1.000$\pm$0, HK+W: 0.601$\pm$0.051[a], LK: 0.724$\pm$0.084[b], B: 1.674$\pm$0.217[c], B+W: 0.774$\pm$0.176[d]. Statistical analysis was done by Student's t test. [a]$P<0.01$, vs. HK, [b]$P<0.05$, vs. HK, [c]$P<0.05$, vs. LK, [d]$P<0.05$, vs. B.



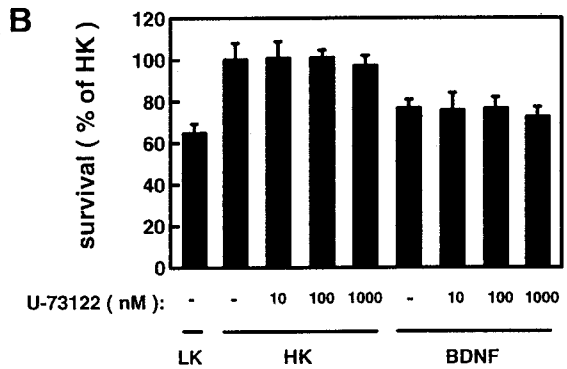


Figure 6. MAP kinase and PLC-γ are not involved in the BDNF- or high K^+-initiated survival pathway in cerebellar granule neurons. The medium was changed to serum-free low K^+ MEM in the absence of BDNF (LK), serum-free low K^+ MEM in the presence of 50 ng/ml BDNF (BDNF) or serum-free high K^+ MEM (HK), without or with each of the inhibitors (Fig. 6A: PD98059, Fig. 6B: U-73122) at indicated concentrations. After 24 hours, the neuronal survival was determined as described in the legend to Figure 1. Values are mean$\pm$S.E.M. (n=3).

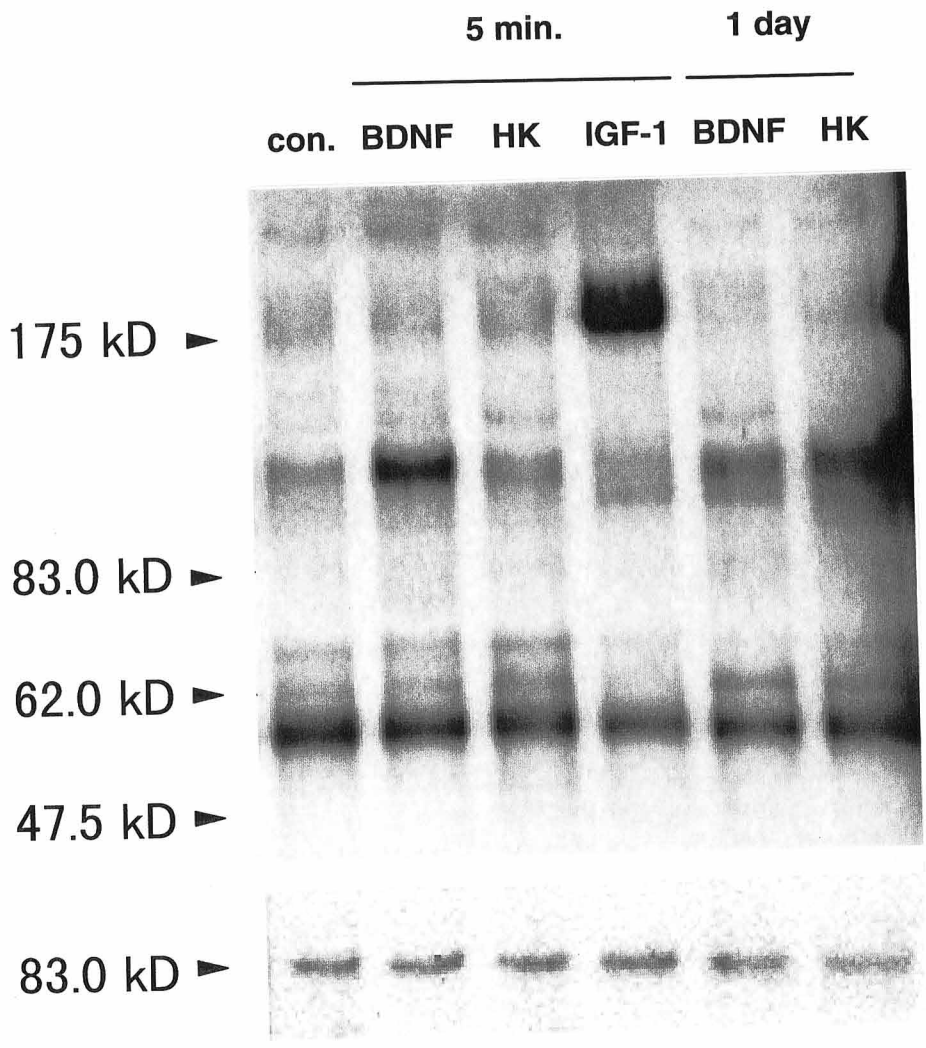


Figure 7. A tyrosine-phosphorylated 95-kD protein binds to PI3-K in response to BDNF and HK$^+$. Cultured cerebellar granule neurons were cultured as described in Materials and Methods. Cells were subjected to serum-free high K$^+$ MEM (HK of 1 day) or serum-free low K$^+$ MEM without (con) or with 50 ng/ml BDNF (BDNF of 1 day) for 1 day. Cells were treated with 50 ng/ml BDNF (BDNF of 5 min) or 100 ng/ml IGF-1 (IGF-1 of 5 min) for 5 minutes in serum-free low K$^+$ MEM, or subjected to serum-free high K$^+$ MEM for 5 minutes (HK of 5 min). Cells were, then, lysed in lysis buffer. Samples were immunoprecipitated with anti-p85 antibody and analyzed as described in **Materials and Methods**.

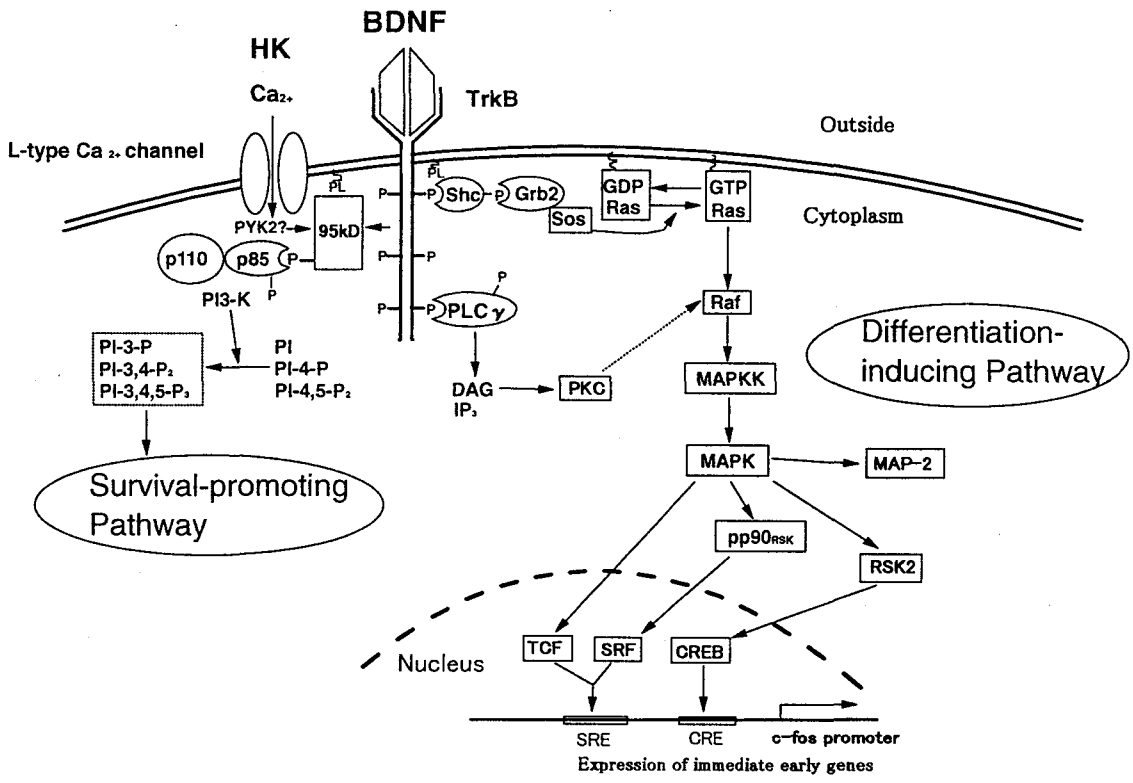


Figure 8. A model for the survival-promoting pathway which is mediated by the activation of PI3-K. The survival signal initiated by BDNF and HK^+ is mediated by PI3-K. PI3-K may be activated by a 95-kD protein. MAPK and PLC-γ are not involved in the survival-promoting pathway (see Discussion for details).

Part 2 :

PI3-Kinase-Akt Pathway Suppresses the Elevation of JNK Activity and Prevents Low K^+-Induced Apoptosis

Abstract

Cerebellar granule neurons die when cultured in serum-free medium containing a low concentration (5 mM) of potassium (LK^+). A high concentration (26 mM) of potassium (HK^+), BDNF and IGF-1 can effectively prevent this apoptotic process. In part 1, I reported that the phosphatidylinositol-3 kinase (PI3-K) pathway which was activated by these agents mediated the prevention of neuronal cell death. Recent reports showed that a serine / threonine kinase, Akt / PKB, was activated by the PI3-K pathway and that LK^+-induced apoptosis was prevented by IGF-1 through Akt / PKB in cerebellar granule neurons. To examine the possibility that Akt can contribute to the survival pathway which is initiated by BDNF or HK^+ in cerebellar granule neurons, the activity of Akt was measured in cells in the condition of LK^+ containing BDNF or in the condition of HK^+, with specific inhibitors of PI3-K, LY294002 and wortmannin. I observed that the PI3-K and Akt activities were decreased when either inhibitor was existed. A synthetic phospholipid derivative, dipalmytoyl-3,4-bisphosphate, which potentially activates Akt, prevented apoptosis in the condition of LK^+. I also observed an increase of the Akt activity in the presence of this synthetic lipid. On the other hand, rapamycin, which suppresses the activation of $p70^{S6K}$, had no effect on the cell viability. These results indicate that two

different survival-promoting stimuli, BDNF and HK^+, can prevent apoptosis of cerebellar granule neurons via the same PI3-K-Akt signaling pathway but not via $p70^{S6K}$. In addition, I also analyzed the signaling pathway downstream of Akt. It is known that the c-Jun N-terminal kinase (JNK) pathway mediates many types of neuronal cell death. I observed that LY294002, a specific inhibitor of PI3-K, increased the JNK activity and the c-Jun expression in the conditions of HK^+ and LK^++ BDNF. These findings demonstrate that the PI3-K-Akt/PKB pathway suppresses the activation of JNK and the c-Jun expression, and prevents the neuronal cell death in cerebellar granule neurons.

Key words:

apoptosis, brain-derived neurotrophic factor (BDNF), high potassium, c-Jun N-terminal kinase (JNK), c-Jun, TrkB, phosphatidylinositol 3-kinase (PI3-K), Akt / PKB, LY294002, wortmannin

1. Introduction

Phosphatidylinositol 3-kinase (PI3-K) is a lipid modifying kinase that phosphorylates the specific 3' position of inositol ring of phosphatidylinositol (Whitman et al., 1988). This enzyme is activated by variety of factors through their cognate receptor-tyrosine kinases, and produces three types of phospholipid, phosphatidylinositol 3-monophosphate (PI-3-P), phosphatidylinositol 3,4-diphosphate (PI-3,4-P_2) and phosphatidylinositol 3,4,5-triphosphate (PI-3,4,5-P_3) (Auger et al., 1989). PI-3,4-P_2 and PI-3,4,5-P_3 are generated transiently by cytokines and survival-promoting factors, but the amount of PI3-P does not change with these agents (Auger et al., 1989). Thus, PI-3,4-P_2 and PI-3,4,5-P_3 may have a crucial role in the signaling pathway downstream of PI3-K (Toker et al.,1994). Recent report revealed that PI-3,4-P_2 had a potential to activate Akt / PKB *in vivo* and *in vitro* (Franke et al., 1997a,b,c). However, the presence of this lipid is not enough to fully activate Akt / PKB, and there is another kinase, termed as PDK1, that activates Akt / PKB completely (Alessi et al., 1997; Stoke et al., 1997; Franke and Cantley, 1997). Akt / PKB is a serine / threonine kinase which may exist in the pathway downstream of PI3-K (Burgering and Coffer., 1995). Recent analysis of cerebellar granule neurons transfected with a dominant negative form of Akt revealed that Akt contributed to the PI3-K-

mediated survival pathway, and that the wild-type Akt prevented apoptosis (Dudek et al., 1997).

On the other hand, PC12 cells which have been differentiated into neuron-like cells by treatment with NGF, die in apoptotic manner when NGF is deprived. Xia and co-workers proved that c-Jun N-terminal kinase (JNK) and p38 MAP kinase (p38 MAPK) (Kawasaki and Kummer, 1997) were activated as an early events in apoptosis after NGF-withdrawal (Xia et al., 1995). Moreover, UV-irradiation (Derijard et al., 1994; Rosette and Karin., 1996) or treatment of tumor necrosis factor (TNF) (Sluss et al, 1994; Natoli et al., 1997), or ceramide (Westwich et al., 1995; Verheij and Cuvillier, 1996) can also activate these molecules and induce apoptosis. It is thought that the activated JNK and p38 MAPK activate the c-Jun protein and then the activated c-Jun increases the c-Jun expression in the nucleus (Derujard, Kyriakis, Sluss, 1994; Virdee et al., 1997; Miller et al., 1997b). However, it is unclear how c-Jun itself leads to the neuronal apoptosis.

As described above, there are two opposite signaling pathways, which one is the apoptosis-preventing pathway (the PI3-kinase-Akt pathway) and the other is the apoptosis-inducing pathway (the JNK or p38 MAPK pathway). Recently, a great progress has been made in the analysis of the former PI3-K pathway, because the specific inhibitors of PI3-K have been developed (Vlahos et al.,1994; Wymann et al., 1996). LY294002 and wortmannin are the two major specific inhibitors of PI3-K, and I chose LY294002 for my

experiments in part 2 because of its stability. Part 1 showed that LY294002 and wortmannin blocked the PI3-K activity and induced apoptosis in the condition of LK^++ BDNF and HK^+. In part 2, I have analyzed the signaling molecules downstream of PI3-K which lead to cell survival. I have observed that the activity of Akt / PKB in the condition of LK^++ BDNF or in the condition of HK^+ is higher than that in the condition of LK^+ alone. A synthetic phosphatidylinositol derivative, PI-3,4-P_2 clearly prevented the LK^+-induced apoptosis. However, I have found that $p70^{S6K}$ which exists in the pathway downstream of Akt is not involved in the survival-promoting effect, from the experiment with an inhibitor of activation of $p70^{S6K}$, rapamycin. These results demonstrate that LK^+-induced apoptosis in cerebellar granule neurons is prevented by the PI3-K-Akt signaling pathway but not by $p70^{S6K}$. I have also observed that LY294002 blocked the PI3-K-Akt pathway and that the activity of JNK was oppositely increased after the addition of LY294002. These results suggest that the PI3-K-Akt pathway prevents the neuronal cell death by blocking the activation of JNK and thus the expression of c-Jun.

2. Materials and Methods

2.1. Cerebellar granule neuron cultures, Hoechst 33258 staining and assays of neuronal survival.

These Methods are described in part 1.

2.2. Immunocytochemistry

For c-Jun staining, cultured cells were fixed with 4 % paraformaldehyde for 20 min and stained with anti-c-Jun/AP-1 antibody (Santa Cruz), the Vectastain ABC kit (Vector Lab.), 0.02 % (w/v) 3,3'-diaminobenzidine 4-HCl (DAB) and 0.1 % (w/v) $(NH_4)_2Ni(SO_4)_2$. For MAP2 staining, cells were incubated with anti-MAP2 antibody (1:5000) overnight at 4 ℃ then stained using the Vectastain ABC kit (Vector Lab.), 0.02 % (w/v) 3,3'-diaminobenzidine 4-HCl (DAB) and 0.3 % H_2O_2.

2.3. In vitro kinase assay

The activity of $p70^{S6K}$ was measured by the S6 Kinase Assay Kit (UBI) with rabbit polyclonal anti-p70 S6K IgG (UBI). Briefly, cells were lysed in

the buffer containing of 1 % Triton X-100, 50 mM Tris-HCl (pH, 7.4), 0.1 % mercaptoethanol, 10 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na_3VO_4, 50 mM NaF, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 1 mM PMSF. Immunoprecipitates with anti-$p70^{S6K}$ were then washed three times with lysis buffer and two times with reaction buffer containing 20 mM MOPS (pH, 7.2), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na_3VO_4, 1 mM DTT and 4 μM PKC and 0.4 μM PKA inhibitor peptide (PKI). Reactions were carried out with a specific substrate (a peptide with the sequence of AKRRRLSSLRA), 5 μM ATP and [γ-^{32}P] ATP (10 μCi / sample) for 20 minutes at 30℃, and the reaction mixtures were spotted onto P81 phosphocellulose papers. Then, these papers were washed with 0.75 % phosphoric acid and acetone. The incorporated radio activities on the papers were quantified by a liquid scintillation counter.

For measurement of the activity of Akt (Dudek et al., 1997), cells were lysed in the buffer containing 1 % NP-40, 10 % glycerol, 137 mM NaCl, 20 mM Tris-HCl (pH, 7.4), 2 μg/ml aprotinin, 1 mM PMSF, 20 mM NaF, 1 mM $NaPP_i$ and 1 mM Na_3VO_4. Immunoprecipitates with anti-Akt antibody (C-20, Santa Cruz) were suspended in the reaction buffer containing 20 mM Hepes (pH, 7.2), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 0.2 mM EGTA, 1 mM DTT and PKI. Reactions were carried out with histone H2B as a substrate, 5 μM ATP and [γ-^{32}P] ATP (10 μCi / sample) for 20 minutes at 30℃ and terminated by addition of the sample buffer. Then, the reaction mixtures

were separated on SDS-PAGE and the incorporation of [^{32}P] into histone H2B was visualized and quantified by Fuji BAS2000.

For measurement of the JNK activity, cells were lysed in lysis buffer containing 20 mM Tris-HCl (pH, 7.4), 1 % Triton X-100, 10 % glycerol, 137 mM NaCl, 1 mM EDTA, 25 mM β-glycerophosphate, 1 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF and 2 μg/ml aprotinin. The insoluble debris were removed by a brief centrifugation. Then, immunoprecipitation was carried out using anti-JNK1 antibody (Santa Cruz), and *in vitro* immune complex kinase reaction was performed as described previously (Khosravi-far et al., 1996) with slight modifications. The samples were then loaded onto SDS-PAGE and the [^{32}P]-labeled products were visualized by autoradiography or by an image analyzer, Fuji BAS2000. The radioactivity was quantified by Fuji BAS2000.

3. Results

3.1. The serine / threonine kinase, Akt / PKB, is involved in the PI3-K-dependent survival pathway in cerebellar granule neurons.

PI3-K has an ability to produce three types of phosphatidylinositol, phosphatidylinositol-3-monophosphate (PI-3-P), phosphatidylinositol-3,4-bisphosphate (PI-3,4-P_2) and phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P_3). These lipids may activate some downstream molecules of PI3-K and mediate the survival-promoting effect. One of the target of PI3-K, Akt / PKB, which has a pleckstrin homology (PH) domain in its N-terminal position, can be the component of the PI3-K-dependent survival pathway because the PH domain can bind to phospholipids and localize Akt to cell membrane. Thus, Akt which is close to the cell membrane may mediate the survival effect. A recent report (Franke et al., 1997a) showed that PI-3,4-P_2 activated Akt directly and PI-3,4,5-P_3 might activate it indirectly, and that both lipids were necessary for the full activation of Akt (Hemmings, 1997; Franke and Cantley, 1997c). However, there was no information about the survival-promoting effect of PI-3,4-P_2 and PI-3,4,5-P_3. I examined whether or not these lipids have the survival effect in cerebellar granule neurons. As shown in Fig. 1, I observed the approximately 30 % of neurons were rescued

in the presence of 5 μM PI-3,4-P_2.

PI-3,4-P_2 has an ability to activate several intracellular molecules (Toker et al., 1994; Franke et al., 1997). To examine whether PI-3,4-P_2 activates the Akt activity or not, the immunocomplex kinase assay using anti-Akt antibody was performed with the cells incubated with PI-3,4-P_2. As shown in Fig. 2, PI-3,4-P_2 indeed increased the Akt activity *in vivo*, suggesting that Akt, which exists in the pathway downstream of PI3-K, is a critical mediator of the survival-promoting effect in cerebellar granule neurons.

To investigate whether Akt is controlled by PI3-K, the Akt activity in the cells treated with PI3-K inhibitors was determined. The Akt activity in the condition of LK^++ BDNF or HK^+ decreased when one of the inhibitors of PI3-K, LY294002 or wortmannin, was added in the medium, and also when the medium was changed to LK^+ (Fig. 3). These results suggest that Akt exists in the downstream of PI3-K and promotes the cell survival in cerebellar granule neurons.

3.2. $p70^{S6K}$ is not important in the survival-promoting effect.

The activation of PI3-kinase is also known to induce the activation of ribosomal S6 kinase ($p70^{S6K}$). Some reports (Weng et al., 1995; Brunn, Klippel, 1996) showed that $p70^{S6K}$ exists in the pathway downstream of Akt. Therefore, $p70^{S6K}$ may act as a survival-promoting component in the signaling

pathway downstream of PI3-kinase. To determine whether $p70^{S6K}$ manifests the survival-promoting effect or not, the activity of $p70^{S6K}$ and the neuronal survival were estimated in the presence of rapamycin, an inhibitor of $p70^{S6K}$ activation. As shown in Fig. 4, the activity of this enzyme was blocked by rapamycin at 500 nM, and wortmannin at 100 nM which inhibited the PI3-K activity. However, the survival-promoting effect of BDNF and high K^+ was not inhibited by rapamycin even at 1000 nM (Fig. 5). These results indicate that $p70^{S6K}$ is not important in the prevention of apoptosis by BDNF and high K^+ in cerebellar granule cells.

3.3. LY294002 increases the c-Jun expression.

The survival mechanisms initiated by BDNF, IGF-1, insulin, bFGF and cAMP have been reported to manifest through at least two pathways, the PI3-K-Akt pathway and an unknown pathway (Franke et al., 1997b). As shown in part 1, HK^+ and BDNF prevented apoptosis through PI3-K. In addition, IGF-1 and insulin prevented apoptosis by the same PI3-K pathway including Akt in the cerebellar granule neurons as reported by other investigators (Dudek et al., 1997; D`Mello et al., 1997; Miller et al., 1997). Miller et al. (1997) showed that cAMP promoted the survival but did not activated PI3-K. On the other hand, Xia. et al. (1995) revealed that the expression of c-Jun, one of the transcriptional factors, was increased in the differentiated PC12 cells

when NGF was deprived. In the case of cerebellar granule neurons, the c-Jun expression was increased in apoptotic conditions (Araki el al., 1998). As shown in Fig. 6, the c-Jun expression was induced when LY294002, an inhibitor of PI3-K, was added to cerebellar granule neurons in the condition of HK^+ or LK^++ BDNF. However, I observed a background increase of expression of c-Jun even in the condition of HK^+ or LK^++ BDNF without LY294002. The stress of serum deprivation may cause the background expression of c-Jun. These results suggest that c-Jun is one of the cell death-inducible molecule, which might induce the transcription of some unknown death genes.

3.4. The JNK activity is increased in the process of LY294002-induced apoptosis.

Ham et al. (1995) showed that the c-Jun expression is achieved through the phosphorylation of c-Jun by Jun N-terminal kinase (JNK) on its amino terminal residues in the early event of cell death. The activation of JNK may mediate the UV-irradiation-, TNF-, Fas-, trophic factor withdrawal-, or staurosporine-induced apoptosis. To examine whether JNK is involved in the process of apoptosis which is induced by inhibitor of PI3-K in cerebellar granule neuron, the activity of JNK was measured in cells cultured in the presence of 10 μM LY294002. As shown in Fig. 7, the activity of JNK

increased transiently 8 hours after changing medium to LK^++ BDNF (without serum) or HK^+ (without serum). In addition, the activity of JNK increased when LY294002 was added to the cultures in the condition of LK^++ BDNF or HK^+. These results demonstrate that PI3-K suppresses the activation of JNK and promotes the cell survival in cerebellar granule neurons (Fig. 8).

4. Discussion

In the nervous system, approximately a half of neurons which are produced by neurogenesis die during development (Oppenheim., 1991). The amount of target-derived neurotrophic factors is not enough for all neurons to survive. Only the neurons which have received sufficient target-derived factors construct the mature nervous system. It is thought that this selection is a much better way to form the accurate neuronal network than the formation of minimum neurons. In the cerebellum, the precursors of granule cells are generated in the external granule cell layer (EGL), and the differentiated granule cells migrate into the internal granule cell layer (IGL) across the molecular and Purkinje cell layers (Burgoyne and Cambray-Deakin, 1988). The cell death of granule cells occurs almost in EGL (Herrup and Sunter, 1987; Wood et al., 1993). In mature neurons, the cell death which is harmful to maintain the biological functions is induced by the exogenous stimuli (Chiueh et al., 1993; Ozaki et al., 1997). This type of cell death is also prevented by trophic factors. Thus, it is important for the human being to elucidate the prevention mechanisms initiated by trophic factors. Recently, the molecular mechanisms of both the cell death and the prevention of cell death have been studied using primary cultured neurons as a model system (D`Mello, Lindholm, 1993; Kubo et al., 1995). I use cerebellar granule

neurons to analyze the prevention mechanisms of cell death because of their abundance and homogeneity. In the previous part of the thesis, I showed that LK^+-induced cell death of cerebellar granule neurons was prevented by PI3-K which was activated by BDNF or HK^+ (Nonomura et al., 1996; Shimoke et al., 1997). PI3-K is a lipid modifying kinase which phosphorylates the D-3 position of inositol ring and activates a variety of signaling molecules, including PKC, Akt/PKB, $p70^{S6K}$ and GSK-3, through its lipid products. PI3-K produces three types of phospholipid, phosphatidylinositol-3-monophosphate (PI-3-P), phosphatidylinositol-3,4-bisphosphate (PI-3,4-P_2) and phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3). PI-3,4-P_2 and PI-3,4,5-P_3, but not PI-3-P, are transiently increased by growth factors and have a potential to activate PKC. In addition, recent reports revealed that a synthetic phospholipid, dipalmitoyl PI-3,4-P_2, activated Akt directly *in vivo* and *in vitro*, and that Akt induced the cell survival in cerebellar granule neurons (Franke et al., 1997a, b; Dudek et al., 1997). Stokoe et al. (1997) and Alessi et al. (1997) showed that PDK1 is necessary for a full activation of Akt through PI-3,4,5-P_3. Other investigator showed that PI3-K and Akt were involved in the survival signal of superior cervical neurons (Philpott et al., 1997). I also show that Akt is activated *in vivo* by the synthetic phospholipid, dipalmitoyl PI-3,4-P_2, and that this lipid significantly prevents the LK^+-induced cell death of cerebellar granule neurons. Moreover, the Akt activity of the cells in the condition of HK^+ or LK^++ BDNF was decreased when the inhibitor of PI3-K,

LY294002 or wortmannin, was added. These results support the observation mentioned in part 1, that PI3-K mediates the cell survival initiated by HK^+ or BDNF.

Akt has a pleckstrin homology (PH) domain in its amino terminal and selectively binds to PI-3,4-P_2 through its PH domain (Franke et al., 1997). PI-3,4,5-P_3 can generate PI-3,4-P_2 in the presence of a 5'-phosphatase and then activate Akt in the same way. As mentioned above, PI-3,4,5-P_3 activates the phosphoinositide-dependent kinase-1 (PDK1), which has a potential to phosphorylate and activate Akt (Allesi et al. and Stokoe et al., 1997). My data cannot neglect the possibility that PDK1 may also activate Akt though PI-3,4,5-P_3 in cerebellar granule neuron. Thus, Akt is activated through at least two pathways and the activated Akt mediates the survival pathway downstream of PI3-K.

I also investigated the role of $p70^{S6K}$ in the survival-promoting effects of HK^+ and BDNF. This kinase phosphorylates the 40S-ribosomal protein S6 and regulates the translation of some transcripts. The function of $p70^{S6K}$ has been studied by an inhibitor of the activation of $p70^{S6K}$, rapamycin, and it has become clear that $p70^{S6K}$ prevents the progression of cell cycle through the G1 phase in mitotic cells. The analysis by the constitutively active or dominant inhibitory mutants of PI3-K revealed that $p70^{S6K}$ is activated through PI3-K (Klippel et al., 1996). The analysis by the constitutive active or dominant inhibitory mutants of Akt also revealed that $p70^{S6K}$ exists in the

pathway downstream of Akt (Burgering et al., 1995; Allesi et al., 1996). Thus, it was likely that this protein might regulate the translation of mRNAs coding for some survival-promoting proteins and promote the cell survival of cerebellar granule neurons. However, rapamycin had no effect on the viability of neurons in the condition of HK^+ or LK^+ + BDNF at any concentrations. Similar results were reported by other investigators (Yao et al., 1996; Gunn-Moor et al., 1997). These findings suggest that some molecules existing in the pathway downstream of Akt might have critical roles in the survival-promotion by HK^+ or BDNF. Dudek et al. (1997) mentioned the possibility that the glycogen synthase kinase-3 (GSK-3), which negatively regulates glycogen synthesis, promotes the cell survival, as the substrate of Akt. However, GSK-3 is activated by many other molecules and is not thought to be the critical molecule for the cell survival. Recently, it has been reported that BAD is phosphorylated on its specific serine residues by Akt, and the phosphorylated BAD is translocated from the mitochondria to the cytosol by binding to the cytosolic protein 14-3-3t. BAD is a member of BCL-2 family, including BCL-2, BAX, BAG-1, HRK, BID, BIK, BCL-X_L, BCL-X_S MCL-1, A1 and BAK. BAD, BAX, HRK, BID, BIK, BAK and BCL-X_S promote the cell death, while BCL-2, BCL-X_L, BAG-1, MCL-1 and A1 promote the cell survival. The functions of the BCL-2 family proteins are achieved by the formation of homo- or heterodimers (Greenlund et al., 1995). A homodimer of BCL-2 or BCL-X_L and a heterodimer of BCL-2 and

BCL-X_L promote the cell survival. On the other hand, a homodimer of BAX promotes the cell death, and the heterodimerization of BAX with BCL-2 or BCL-X_L neutralizes the cell death-promoting function of BAX. BAD also interacts with BCL-2 or BCL-X_L . A series of the interactions among BAX, BCL-2, BCL-X_L and BAD is equilibrated on the mitochondrial membrane. The phosphorylation of BAD through Akt dissociates BAD from BCL-2 or BCL-X_L, and induces the formation of a homo- or heterodimer of BCL-2 and BCL-X_L which promote the cell survival. The other possible mechanism of the cell survival through the PI3-K-Akt pathway is that the expression of BCL-2 protein might be increased by the PI3-K-Akt signaling (Ahmed et al., 1997).

On the other hand, the cell death is mediated by the cell death-inducing signal. The cell-death signal can be divided into three types of signal, the phosphorylation-mediated signal, the protease-mediated signal and the DNA damage-mediated signal. With respect to the phosphorylation-mediated signal, Xia et al. (1995) reported that JNK and p38 MAP kinase are involved in the cell death of PC12 cells induced by NGF withdrawal. JNK is activated through ceramide by the TNF receptor, Fas, p75 neurotrophin receptor or X-ray irradiation. Thus, I examined whether or not JNK is involved in the cell death of cerebellar granule neurons. As expected, I observed that JNK was activated when LY294002, an inhibitor of PI3-K, was added to the condition of HK^+ or LK^++ BDNF. In addition, c-Jun was expressed at nearly the same

time as the JNK activation. Watson et al. (1998) also showed that the phosphorylation of c-Jun is critical process of the cell death induced by trophic factor withdrawal, and that the level of c-jun mRNA and protein increases in the condition of cell death. They mentioned that p38 MAP kinase is also activated but the magnification of activation was lower than that of the JNK. These results support my data that the survival signal mediated by PI3-K suppresses the JNK activity and the c-Jun expression in cerebellar granule neurons. In SCG neurons, Philpott et al. (1997) showed that c-Jun is not involved in the survival signal by using dominant inhibitory- or constitutively active mutants of PI3-K. The involvement of the suppression of c-Jun in the survival signal may be cell-type specific. Nobody demonstrated that the PI3-K-Akt signal is directly involved in the protease-mediated signal or the DNA damage-mediated signal.

In summary, I have investigated the role of the PI3-K-Akt signaling pathway in the survival promotion of cerebellar granule neurons. This pathway suppresses the elevation of the JNK activity and the c-Jun expression (Fig. 8). According to the result by Deshmukh et al. (1996), there are four stages in the cell death, the activation stage, the propagation stage, the commitment stage and the execution stage. The activation stage includes the production of reactive oxygen species (ROS) and the JNK activation, and the propagation stage includes the c-Jun expression, and the commitment stage includes the BAX activity. In the final execution stage, the activation of caspase cascade,

the DNA fragmentation and the chromatin condensation are observed. My and other investigator`s data indicate that the survival signal initiated by trophic factors and HK^+ suppresses the cell death-signal at least two stages, the activation and the commitment stages. Because the mechanism of the cell survival initiated by trophic factor and HK^+ has become clearer by my work, it is possible to take advantage of my results in the therapeutics of the neuronal cell death in future.

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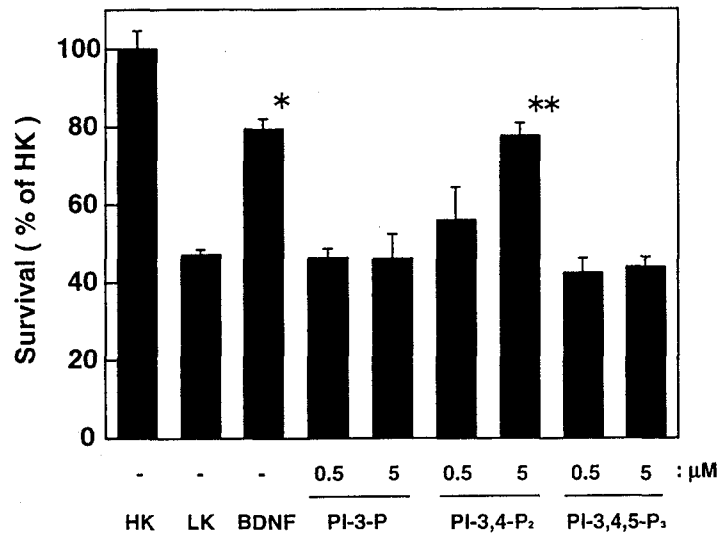

Figure 1. Effects of synthetic phospholipids, PIP, PIP_2 and PIP_3, on the cell survival. The medium was changed to serum-free MEM in the absence (LK) or presence of BDNF (50 ng/ml) (BDNF) or synthetic phopholipids (PIP, PIP_2 and PIP_3) at indicated concentrations, or serum-free high K^+ (26 mM) MEM (HK). After 24 hours, the neuronal survival was determined by the MTT assay as described in **Materials and Methods**. The mean value obtained from cells maintained in serum-free high K^+ MEM (HK) was defined as the control. Values are means ± S.E.M. (n=3). Statistical analysis was performed by Student's t test. $^*P<0.001$, vs. LK and $^{**}P<0.001$, vs. LK.

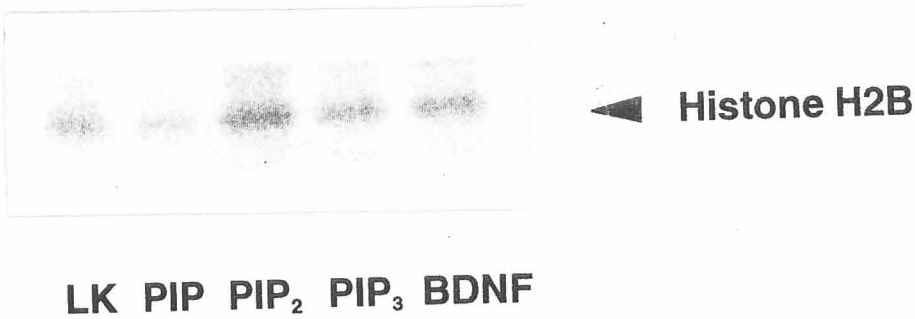

Figure 2. Akt is activated by a synthetic phospholipid, PIP_2, *in vivo*. Cells were cultured in serum-free MEM for 1 overnight, and synthetic phopholipids were added to the cultures at 10 μM for 10 minutes. Then, cells were lysed in lysis buffer and the immunocomplex *in vitro* kinase assay was carried out using histone H2B as the substrate, as described in **Materials and Methods**. The radioactivity incorporated into histone H2B was visualized by BAS2000 (Fuji Film). The densitometry was also performed using BAS2000. Relative intensities were as follows. LK: 1.00, PIP: 0.61, PIP2: 1.82, PIP3: 1.22, BDNF: 1.36.

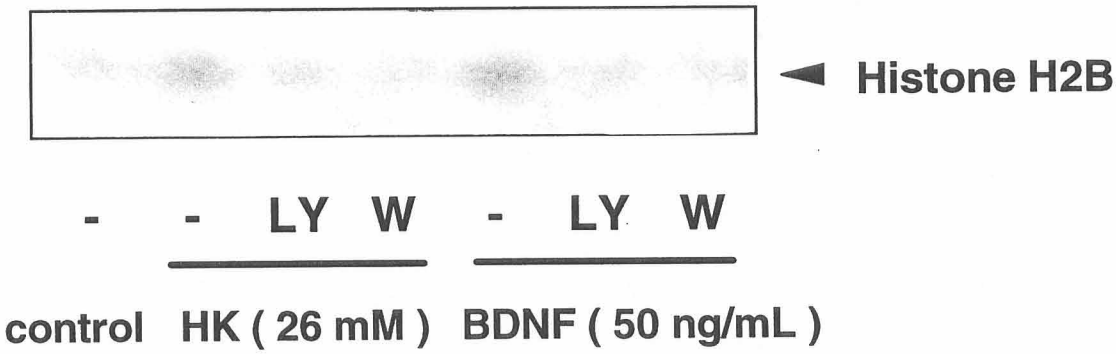

Figure 3. Akt activated by BDNF and HK^+ is inhibited by LY294002 and wortmannin *in vivo*. Cerebellar granule neurons were maintained as **Material and Methods**. Cells were subjected to serum-free HK^+ MEM (HK) or serum-free MEM without (control) or with 50 ng/ml BDNF (BDNF) for 1 day. The activity was estimated 30 minutes after the addition of 10 μM LY294002 (LY) or 100 nM wortmannin (W) by the *in vitro* kinase assay using histone H2B as the substrate. The densitometry was done by Fuji BAS2000 and the relative activities were estimated as follows. control: 1.00, HK (-): 1.17, HK (LY): 1.01, HK (W): 0.91, BDNF (-): 1.17, BDNF (LY): 0.99, BDNF (W): 0.88. This result is representative of two independent experiments.

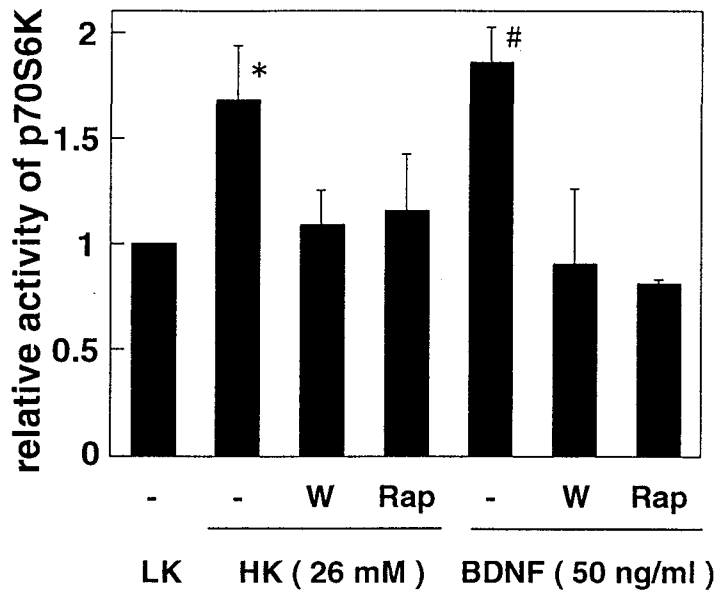

Figure 4. The p70^{S6K} activity was blocked by rapamycin and wortmannin. Cultured cerebellar granule neurons were maintained as **Material and Methods**. Cells were subjected to serum-free HK^{+} MEM (HK) or serum-free MEM without (LK) or with 50 ng/ml BDNF (BDNF) for 1 day. The activity was measured 30 minutes after the addition of 100 nM wortmannin (W) or 500 nM rapamycin (Rap), as described in **Materials and Methods**. Relative activities (means$\pm$S.D., n=3) were calculated and statistical analysis was performed by Student's t-test. $^{*}P<0.05$, vs. HK+W, $^{\#}P<0.05$, vs. BDNF+W.

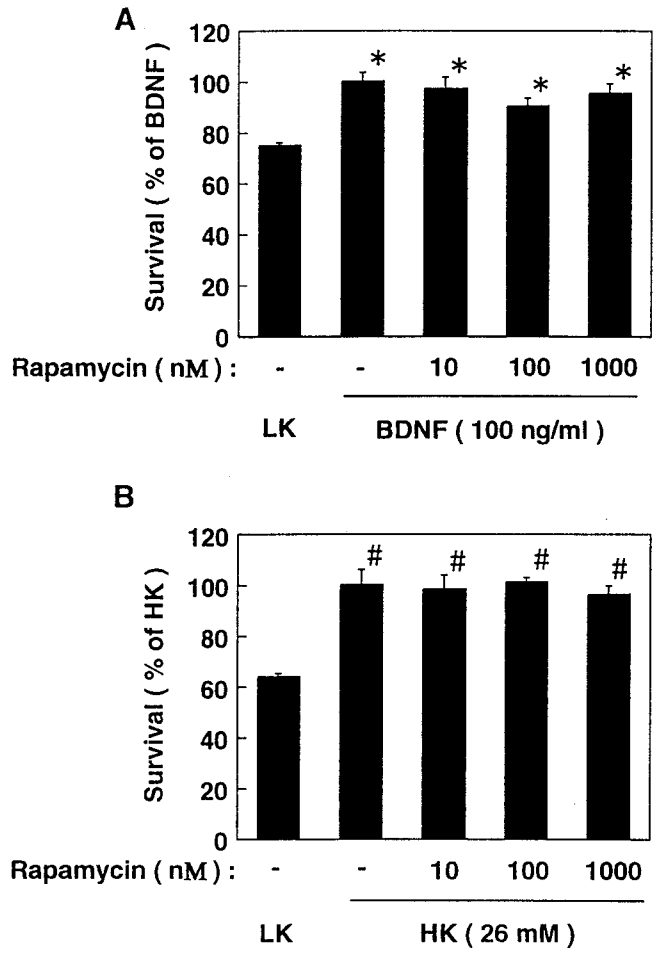

Figure 5. Rapamycin does not block the survival-promoting effect of BDNF and high K^+. The medium was changed to serum-free MEM in the absence (LK) or presence of BDNF (100 ng/ml) (A), or serum-free high K^+ MEM (B), then rapamycin (Rap) was added to the cultures at indicated concentrations (nM). After 24 hours, the neuronal survival was determined as described in **Materials and Methods**. Values are mean± S.E.M. (n=3). Statistical analysis was performed by ANOVA. $^*P<0.001$, vs. none, $^\#P<0.001$, vs. LK. These results are representative of three independent experiments.

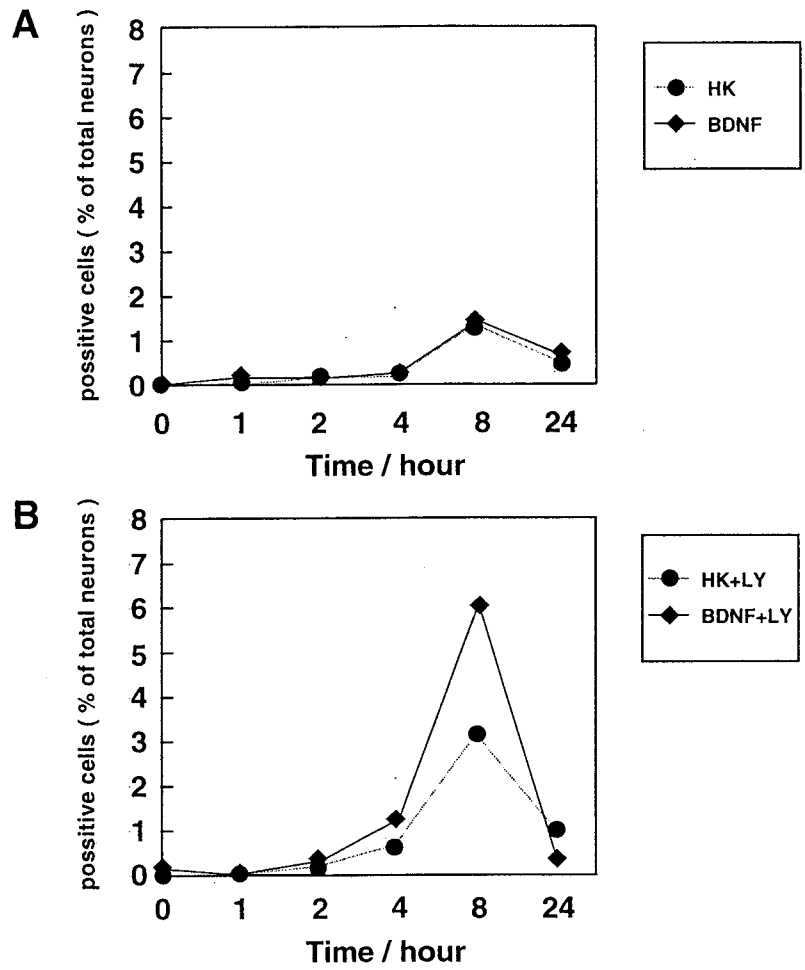

Figure 6. The increased expression of c-Jun by LY294002. Cells were cultured in serum-free MEM in the presence of BDNF (50 ng/ml) (BDNF) or serum-free high K^+ (26 mM) MEM (HK), without or with 10 μM LY294002 (added 30 minutes before the addition of BDNF or at the same time as the medium change to HK)(BDNF+LY, HK+LY), for indicated hours. After the incubation, cells were fixed with 4 % parahormaldehyde and treated with 0.1 % Triton X-100 and 0.1 % sodium citrate solution for 10 min. Anti-c-Jun/AP-1 antibody (Santa Cruz) was added to the mixtures, and then they were incubated at 4℃ for 2 days. Cells were stained by the Vectastain ABC kit (Vector Lab.) as described in **Materials and Methods**. The possitive cells with anti-c-Jun/AP-1 antibody and anti-MAP-2 antibody were counted and relative percentages of possitive cells in total cells were estimated.

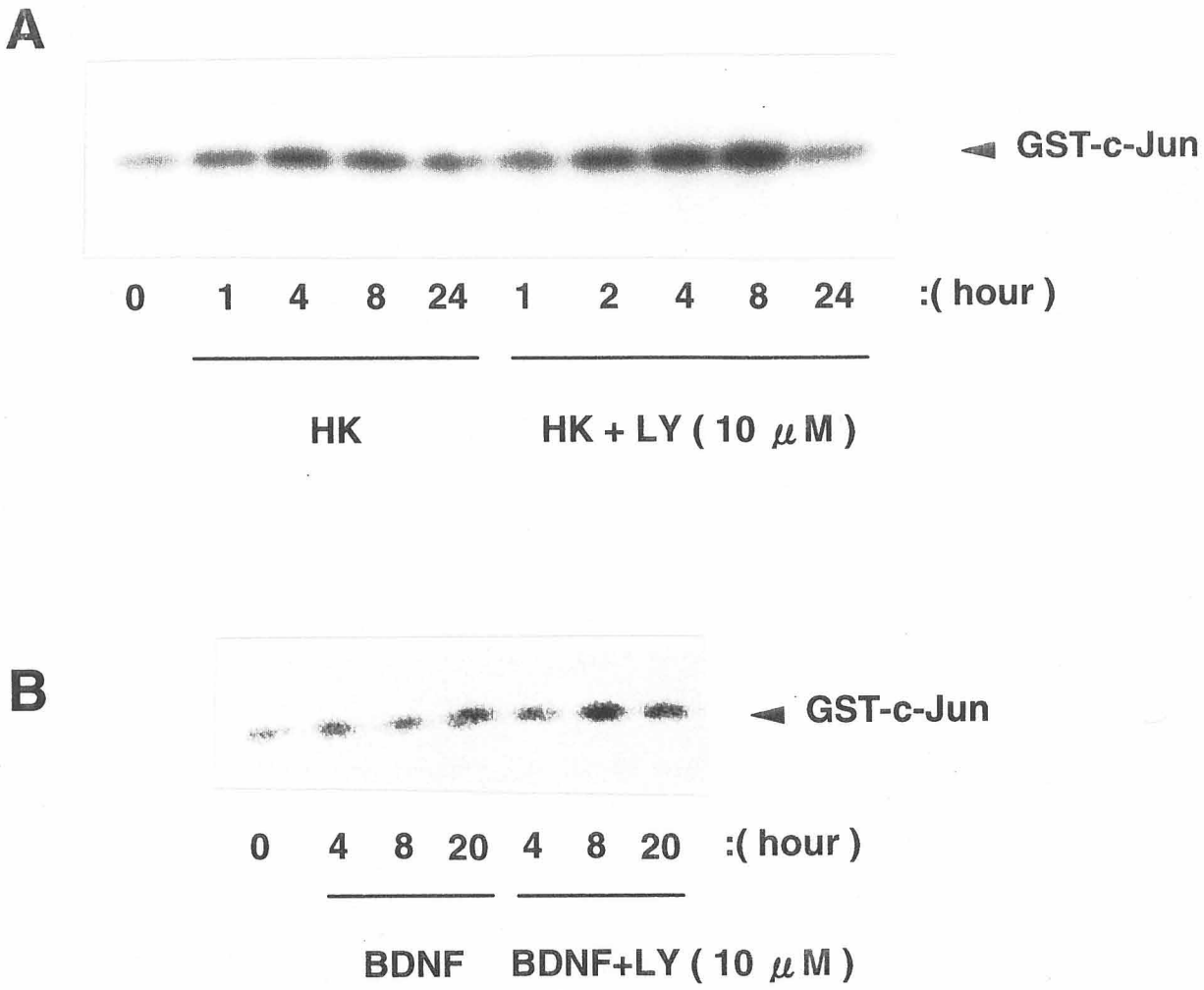

Figure 7. The activity of JNK is stimulated by LY294002. Cells were incubated in serum-free MEM in the presence of BDNF (50 ng/ml) (BDNF) or serum-free high K^+ (26 mM) MEM (HK), without or with 10 μM LY294002 (added 30 minutes before the addition of BDNF or at the same time as the medium change to HK)(BDNF+LY, HK+LY), for indicated hours. The JNK activity was estimated as described in **Materials and Methods.** Cells were lysed in lysis buffer and imunoprecipitation with anti-JNK1 antibody was carried out. The radioactivity incorporated onto GST-c-Jun was visualized by autoradiography or BAS2000 (Fuji Film). The densitometry was performed using BAS2000. Relative intensities were as follows. A, HK (0: 1.00, 1: 1.47, 4: 1.76, 8: 1.58, 24: 1.49), HK+LY (1: 1.34, 2: 1.81, 4: 1.91, 8: 2.42, 24: 1.43). B, BDNF (0: 1.00, 4: 1.18, 8: 1.14, 20: 1.36), BDNF+LY (4: 1.20, 8: 1.39, 20: 1.30).

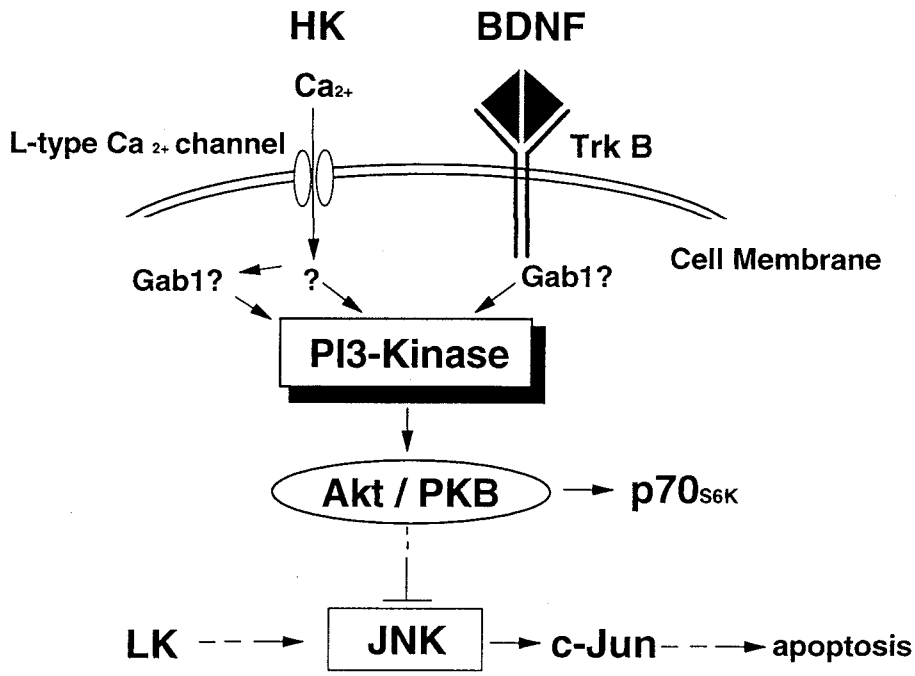

Figure 8. A schematic representation of the survival-promoting pathway initiated by HK^+ and BDNF. PI3-K is activated by HK^+ and BDNF, and the downstream molecules are activated in turn via PIP_2 as a second messenger. $p70^{S6K}$ is excluded from this pathway. The PI3-K-Akt pathway suppresses the activation of JNK and the expression of c-Jun (see Discussion for details).

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