Synthetic lipid products of PI3-kinase which are added to culture medium prevent low K^+-induced apoptosis of cerebellar granule neurons via Akt kinase activation

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Abstract To examine which lipid product of phosphatidylinositol 3-kinase (PI3-K) is essential for the survival-promoting pathway in cultured cerebellar granule neurons, three synthetic derivatives of lipid products of PI3-K were added to culture medium containing a low concentration (5 mM) of potassium (LK^+) which induces apoptotic cell death. We found that dipalmitoylphosphatidylinositol 3,4-bisphosphate and dipalmitoylphosphatidylinositol 3,4,5-trisphosphate, but not dipalmitoylphosphatidylinositol 3-monophosphate, effectively blocked the LK^+-induced apoptosis. These two synthetic phospholipids increased Akt activity but not that of PI3-K. These findings demonstrated that specific lipid products of PI3-K which are added to culture medium activate Akt/PKB without modulating PI3-K itself, and as a result prevent neuronal cell death in cerebellar granule neurons.

Key words: Akt kinase; Apoptosis; Phosphatidylinositol 3-kinase; Phosphatidylinositol 3,4-bisphosphate; Phosphatidylinositol 3,4,5-trisphosphate; Survival signal

1. Introduction

The molecular mechanisms of both neuronal apoptosis and survival have been analyzed using in vitro model systems. We used cultured rat cerebellar granule neurons because of their abundance and homogeneity. A low potassium concentration (LK^+) in the medium induces apoptosis of cerebellar granule neurons, and this apoptosis is prevented by a high potassium concentration (HK^+), BDNF, IGF-1, bFGF or cAMP [1–3]. Previously, we showed that the LK^+-induced apoptosis of cerebellar granule neurons was prevented by BDNF or HK^+ through phosphatidylinositol 3-kinase (PI3-K) [4,5].

PI3-K is a lipid-modifying kinase that specifically phosphorylates the 3′ position of the inositol ring of phosphatidylinositol [6–9]. This enzyme is activated by a variety of receptor-tyrosine kinases, and produces three types of phospholipid, phosphatidylinositol 3-monophosphate (PtdIns-3-P), phosphatidylinositol 3,4-diphosphate (PtdIns-3,4-P_2) and phosphatidylinositol 3,4,5-triphosphate (PtdIns-3,4,5-P_3) [10]. PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 are generated transiently by cytokines and survival-promoting factors, but the amounts of PtdIns-3-P do not change in response to these agents [11]. Thus, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 may have crucial roles in the signaling pathway downstream of PI3-K [12]. Recent studies revealed that PtdIns-3,4-P_2 activated the enzymatic activity of Akt/PKB in vivo and in vitro [13,14]. Akt/PKB is a serine/threonine kinase which may function in the pathway downstream of PI3-K [15]. However, presence of PtdIns-3,4-P_2 alone is not sufficient to fully activate Akt/PKB, and there are other kinases, PDK1 and 2, that activate Akt/PKB completely [14,16,17]. Recent analysis of cultured cerebellar granule neurons revealed that Akt contributes to the PI3-K-mediated survival pathway, and that the wild-type Akt prevented apoptosis [18].

Recently, some progress has been made in analysis of the PI3-K pathway, because specific inhibitors of PI3-K, LY294002 and wortmannin, have been developed [19,20]. Previously, we showed that wortmannin blocked the PI3-K activity and induced apoptosis in cultured cerebellar granule neurons [5]. We and others have analyzed the signaling molecules downstream of PI3-K which lead to cell survival ([18,21] and Shimoke et al., submitted). Our results indicated that the activity of Akt/PKB under LK^++BDNF or HK^+ conditions is higher than that under LK^+ conditions alone (Shimoke et al., submitted). In this study, addition of synthetic derivatives of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 to culture medium clearly prevented the LK^+-induced apoptosis of cerebellar granule neurons through the activation of Akt kinase. These results suggested that direct activation of Akt kinase by synthetic phospholipids which are supplied from culture medium prevents death of cerebellar granule neurons.

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Abbreviations: PI3-K, phosphatidylinositol 3-kinase; HK^+, high potassium; LK^+, low potassium; BDNF, brain-derived neurotrophic factor; diC_{16}, dipalmitoyl; MEM, minimum essential medium; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; PDK1, phosphoinositide-dependent kinase-1; PH, pleckstrin homology; PKB, protein kinase B; $p70^{S6K}$, p70 ribosomal S6 kinase; PtdIns-3-P, phosphatidylinositol 3-monophosphate; PtdIns-3,4-P_2, phosphatidylinositol 3,4-diphosphate; PtdIns-3,4,5-P_3, phosphatidylinositol 3,4,5-triphosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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 previously [1–5,22,23]. After 4 days in culture, the medium was changed to serum-free LK^+ (5 mM potassium) MEM or serum-free HK^+ (26 mM potassium) MEM. At the same time, synthetic phospholipids, diC_{16}PtdIns-3-P, diC_{16}PtdIns-3,4-P_2 and diC_{16}PtdIns-3,4,5-P_3 (Matreya, Inc.), were added to LK^+ medium at the indicated concentrations.

2.2. Assay of neuronal survival

Neuronal survival was determined by the MTT assay [2,5]. A modification [24] of the original procedure [25] was used to measure mi-

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tochondrial function. Briefly, the tetrazolium salt MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) was added to cultures (final concentration of 1 mg/ml). After a 2-h incubation at 37°C, the reaction was stopped by adding lysing buffer (20% SDS in 50% aqueous *N*,*N*-dimethylformamide solution, pH 4.7). The absorbance was measured spectrophotometrically at 570 nm after a further overnight incubation at 37°C. The percent survival was defined as [Abs.(experimental−blank)/Abs.(control−blank)]×100, and the blank was the value taken from wells without cells.

2.3. In vitro kinase assay

The PI3-K activity was measured as described previously [5,26,27] with slight modifications.

For measurement of the activity of Akt [18], cells were lysed in 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. Synthetic phospholipids, diC_{16}PtdIns-3-P, diC_{16}PtdIns-3,4-P_2 and diC_{16}PtdIns-3,4,5-P_3 (Matreya, Inc.), were added in the medium 10 min before lysing if necessary. The protein concentrations of lysates were determined by means of the BCA protein assay. Immunoprecipitates with anti-Akt antibody (C-20, Santa Cruz) were suspended in 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 (500 ng) as a substrate, 5 μM ATP and [γ-^{32}P]ATP (10 μCi/sample) for 20 min at 30°C and terminated by addition of sample buffer. Then, the reaction mixtures were separated by SDS-PAGE, and the incorporation of ^{32}P into histone H2B was visualized and quantified using a Fuji BAS2000 bioimage analyzer.

3. Results and discussion

PI3-K produces three types of phosphatidylinositol, PtdIns-3-P, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3. These lipids may activate some downstream molecules of PI3-K and mediate the survival-promoting effect [28]. One of the targets of PI3-K, Akt/PKB, which has a pleckstrin homology (PH) domain in its N-terminus, can be a component of the PI3-K-dependent survival pathway because the PH domain can bind to phospholipids and cause localization of Akt to the cell membrane. Thus, Akt which is close to the cell membrane may mediate the survival-promoting effect of PI3-K. Recent studies showed that PtdIns-3,4-P_2 activated Akt directly, that PtdIns-3,4,5-P_3 might have an indirect activating effect, and that both lipids were necessary for the full activation of Akt [13,14,29]. Recently, the mechanism of Akt activation by two lipid-dependent kinases, PDK1 and PDK2, has been mentioned [30]. Akt is localized to the cell membrane by binding to specific phospholipids PtdIns-3,4-P_2 and/or PtdIns-3,4,5-P_3 through its PH domain [13,14]. PDK2 activated by PtdIns-3,4,5-P_3 phosphorylates specific serine/threonine residues of Akt in addition to phosphorylation by PDK1 [30].

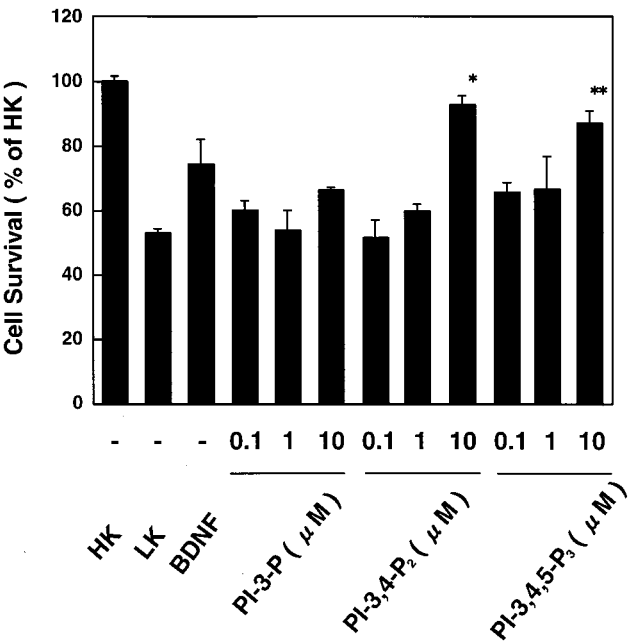

Fig. 1. Synthetic phospholipids promote survival in LK$^+$-induced apoptosis of cerebellar granule neurons. Cerebellar granule neurons were cultured as described in Section 2. After 4 days in culture, the medium was changed to serum-free LK$^+$ MEM (LK) as a control. At the same time, synthetic phospholipids, diC_{16}PtdIns-3-P (PI-3-P), diC_{16}PtdIns-3,4-P_2 (PI-3,4-P_2) and diC_{16}PtdIns-3,4,5-P_3 (PI-3,4,5-P_3) (Matreya, Inc.), were added to LK at the indicated concentrations. After 1 day, the cell viability was measured with the MTT assay as described in Section 2. Values are means ± S.D. ($n=3$). Statistical analysis was performed with Student's *t*-test. *$P<0.001$, vs. LK and **$P<0.001$, vs. LK.

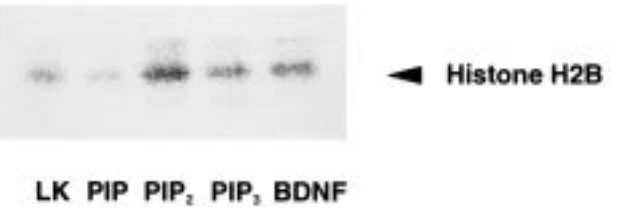

Fig. 2. Synthetic phospholipids activate Akt in intact cerebellar granule neurons. Cerebellar granule neurons were cultured as described in Fig. 1. After 4 days in culture, the medium was changed to serum-free LK$^+$ MEM (LK), cells were cultured for 1 day, and synthetic phospholipids, diC_{16}PtdIns-3-P (PIP), diC_{16}PtdIns-3,4-P_2 (PIP_2) and diC_{16}PtdIns-3,4,5-P_3 (PIP_3), were added to LK at 10 μM for 10 min. Then, immunocomplex kinase assay was carried out using anti-Akt antibody and the incorporated radioactivity bound to histone H2B as the substrate was visualized using a Fuji BAS2000 bioimage analyzer. Densitometric analysis was performed and relative intensities were estimated as follows. LK: 1.0; PIP: 0.6; PIP_2: 1.8; PIP_3: 1.2; BDNF: 1.4. Results are representative of three independent experiments.

However, no information is available about the survival-promoting effects of PtdIns-3-P, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3. We examined whether these lipids have survival-promoting effects in cerebellar granule neurons using the synthetic derivatives diC_{16}PtdIns-3-P, diC_{16}PtdIns-3,4-P_2 and diC_{16}PtdIns-3,4,5-P_3. As shown in Fig. 1, we observed that LK$^+$-treated neurons were rescued to nearly the same levels as under HK$^+$ conditions in the presence of 10 μM diC_{16}PtdIns-3,4-P_2 and diC_{16}PtdIns-3,4,5-P_3, but not with diC_{16}PtdIns-3-P.

DiC_{16}PtdIns-3,4-P_2 activates several intracellular molecules [12,13]. To examine whether diC_{16}PtdIns-3,4-P_2 and diC_{16}PtdIns-3,4,5-P_3 activate Akt activity, an immunocomplex kinase assay using anti-Akt antibody was performed with cells treated with diC_{16}PtdIns-3,4-P_2 or diC_{16}PtdIns-3,4,5-P_3. As shown in Fig. 2, both diC_{16}PtdIns-3,4-P_2 and diC_{16}PtdIns-3,4,5-P_3, but not diC_{16}PtdIns-3-P, increased Akt activity in vivo, suggesting that Akt, which has a role in the pathway downstream of PI3-K, promotes survival of cerebellar granule neurons. As a control, Akt activity in the cells treated with BDNF was determined. BDNF, as well as HK$^+$ (Shimoke et al., submitted), increased Akt activity to the same level as diC_{16}PtdIns-3,4,5-P_3, consistent with its survival-promoting effect on cerebellar granule neurons (Fig. 2).

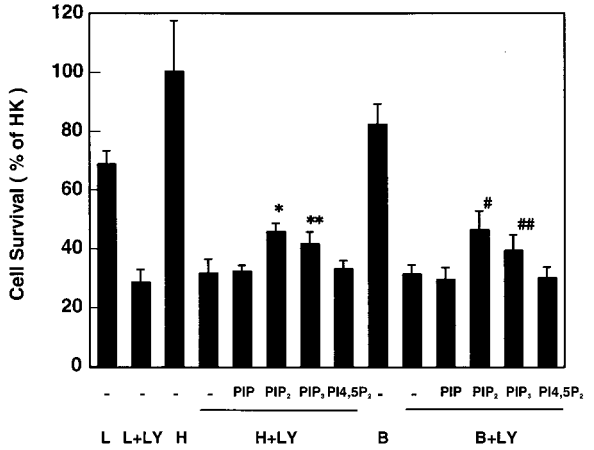

Fig. 3. Synthetic phospholipids promote survival in LY294002-induced apoptosis of cerebellar granule neurons. Cerebellar granule neurons were cultured and the cell viability was measured as described in Fig. 1. After 4 days in culture, the medium was changed to serum-free LK^+ (L), serum-free HK^+ (H) or LK^++50 ng/ml BDNF (B). LY294002 was added to L (L+LY), H (H+LY) and B (B+LY), and at the same time synthetic phospholipids (PIP, PIP_2, PIP_3) were added to H+LY and B+LY at 10 μM. After 1 day, the cell viability was measured with the MTT assay as described in Section 2. PI4,5P2 indicates phosphatidylinositol 4,5-bisphosphate (Sigma). Values are means ± S.D. ($n = 3$). Statistical analysis was performed with Student's t-test. $^*P < 0.01$, vs. H+LY. $^{**}P < 0.01$, vs. H+LY. $P < 0.01$, vs. B+LY. $P < 0.05$, vs. B+LY.

Although LK^+ in the medium induces apoptosis through the suppression of PI3-K activity [5], inhibition of PI3-K by the specific inhibitors LY294002 and wortmannin also induces apoptosis in cerebellar granule neurons ([5,31] and Shimoke et al., submitted). To compare the death-inducing pathways of LK^+- and LY294002-induced apoptosis in cerebellar granule neurons, we examined the effects of synthetic phospholipids on the latter. As shown in Fig. 3, a significant percentage of LY294002-treated neurons were also rescued by 10 μM synthetic diC_{16}PtdIns-3,4-P_2 and diC_{16}PtdIns-3,4,5-P_3, but not by diC_{16}PtdIns-3-P. These results suggested that the death signals induced by LK^+ and LY294002 have a common pathway which is prevented by Akt kinase activation. The observation that LY294002-treated neurons were only partially rescued by synthetic phospholipids implicates LY294002-specific pathways other than the common pathway (inhibition of PI3-K activity) in the LY294002-induced death signal. In fact, when LY294002 was added to culture medium in LK^+, it induced an additive death-inducing effect on LK^+-treated cerebellar granule neurons (Fig. 3).

To investigate whether synthetic phospholipids affect PI3-K activity by an unknown feedback regulatory mechanism [27], PI3-K activity was measured in cerebellar granule neurons treated with synthetic phospholipids. These synthetic phospholipids did not increase the PI3-K activity itself (data not shown). These observations indicated that specific lipid products of PI3-K activate Akt/PKB without modulating PI3-K itself, and as a result prevent death of cerebellar granule neurons.

Addition of diC_{16}PtdIns-3,4-P_2 and diC_{16}PtdIns-3,4,5-P_3 to culture medium similarly promotes cell survival of cerebellar granule neurons via the activation of Akt kinase. Our results suggest that these synthetic phospholipids may be incorporated into the cell membrane from culture medium, and Akt may be localized to the cell membrane by binding to these lipids and be activated (phosphorylated) by phosphoinositide-dependent kinases (PDK1 and 2) [16,17,30]. Although how the synthetic phospholipids are incorporated into the cell membrane from culture medium is at present unclear, our finding provides a simple method to estimate the role of the PI3-K-Akt pathway and to identify which lipid product of PI3-K is essential, in many biological responses of various types of cells. In this context, our observation indicates that PtdIns-3-P is not essential for either survival promotion or Akt kinase activation, in cultured cerebellar granule neurons.

We and others have demonstrated that $p70^{S6K}$, which plays a role in the pathway downstream of Akt, is not involved in the survival-promoting effect of PI3-K, based on the results of experiments with an inhibitor of activation of $p70^{S6K}$, rapamycin [5,32]. These results demonstrated that LK^+-induced apoptosis in cerebellar granule neurons is prevented by the PI3-K-Akt signaling pathway but not by $p70^{S6K}$. Crowder and Freeman [21] reported that Akt has also an important role in cell survival of sympathetic neurons. On the other hand, Datta et al. [33] reported that direct phosphorylation of the cell death inducer BAD by Akt kinase [34] is correlated with survival of cerebellar granule neurons. Taken together, these observations indicate that the PI3-K-Akt pathway prevents neuronal cell death by activating an unknown signaling molecule other than $p70^{S6K}$ and neutralizing the death-promoting function of BAD.

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