Cultured rat cerebellar granule neurons are widely used as a model system for studying neuronal apoptosis. After maturation by culturing in medium containing 26 mM potassium (high K⁺), changing to medium containing 5 mM potassium (low K⁺); LK rapidly induces neuronal apoptosis. Then over 50% of granule cells die within 24 h. However, the molecular mechanisms by which the LK-induced apoptosis occurs in cultured cerebellar granule cells remain unclear. In the present study, we found that p38 MAP kinase (p38) was an important factor for LK-induced apoptosis. Three hours after changing to LK medium, p38 was markedly activated. In addition, SB203580, a specific inhibitor of p38, strongly inhibited the phosphorylation and expression of c-Jun in LK-induced apoptosis of cultured cerebellar granule cells. In vitro kinase assay using glutathione S-transferase-c-Jun as a substrate showed that p38 directly phosphorylated c-Jun. Furthermore, in the presence of SB203580, about 80% of neurons survived. These results indicate that p38 regulates LK-induced apoptosis of cerebellar granule neurons.

Apoptosis is a fundamental biological process used to eliminate unwanted, superfluous, or potentially harmful cells. In the developing nervous system, about half of all neurons that are produced die by apoptosis around the time of birth (1). Cultured rat cerebellar granule cells are widely used as a model system for studying neuronal apoptosis. These neurons are usually cultured and matured in medium containing 26 mM potassium (high K⁺); HK). After maturation, changing to medium containing 5 mM potassium (low K⁺); LK) induces neuronal cell death in which the cells show apoptotic features. In addition, this culture system provides a large homogeneous neuronal population. Therefore, these neurons are widely used as a primary cell culture system to investigate the biochemical and molecular mechanisms underlying neuronal apoptosis in the central nervous system.

Mitogen-activated protein (MAP) kinases are serine/threonine kinases that play an important role in signal transduction from the cell surface to the nucleus. The mammalian MAP kinases can be subdivided into extracellular signal-regulated kinases, c-Jun N-terminal kinases (JNK), and p38 MAP kinases (p38). p38 is activated by phosphorylation on Thr-180 and Tyr-182 in response to environmental stress (2, 3). Recently, p38 activation has been suggested to be involved in mediating apoptosis in various cell types (4–6). SB203580, a specific inhibitor of p38, prevents several types of cell death, including glucamate-induced apoptosis of cultured cerebellar granule neurons (7, 8).

c-Jun is a member of the activator protein-1 family of transcription factors possessing leucine zippers. c-Jun is phosphorylated on Ser-63 and Ser-73 within its N-terminal region by JNK (9). Phosphorylation of c-Jun is necessary for apoptosis of superior cervical ganglion and cerebellar granule neurons (10, 11). However, the mechanism of c-Jun phosphorylation remains unclear.

In the present study, we investigated whether p38 is necessary for LK-induced apoptosis and whether p38 is involved in the phosphorylation of c-Jun in cerebellar granule neurons. Our results suggest that p38 was activated after lowering potassium concentration, and active p38 phosphorylates c-Jun directly. We found the p38-c-Jun pathway is very important for LK-induced apoptosis in cerebellar granule neurons.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary cultures of dissociated cerebellar granule neurons were prepared from the cerebella of postnatal day 9 rats (Wistar ST, both sexes) as described previously (12–16). Briefly, cells were gently dissociated with a plastic pipette after digestion with papain (90 units/ml, Worthington) at 37 °C. The cells were then cultured in medium consisting of 5% precolostrum newborn calf serum (Mitsubishi Kasei), 5% heat-inactivated horse serum (55 °C, 30 min; Life Technologies, Inc.), and 90% 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium containing 15 mM HEPES buffer (pH 7.4), 30 mM sodium bicarbonate, and 1.9 mg/ml sodium bicarbonate. After culture for 1 day in a humidified CO₂ (5%) incubator, the medium was changed to 26 mM potassium-containing (HK) minimal essential medium (MEM) supplemented with 5% heat-inactivated horse serum and 1 μM cytisine arabinoside. MEM was supplemented with 2.2 mg/ml glucose and 2.2 mg/ml sodium bicarbonate. HK-MEM was prepared by increasing the KHCO₃ concentration from the normal low value of 5.4 to 26 mM, with the omission of the corresponding concentration NaHCO₃. After 4 days in culture in a 10% CO₂ incubator, the medium was changed to serum-free 5.4 mM potassium-containing (LK) MEM or HK-MEM. The assays described below were then performed.
protein concentration was determined using a BCA protein assay kit (pH 8.0), 10 mM NaF, 2 mM Na₃VO₄, 0.5 mM phenylarsine oxide, 10 mM kinase reaction was carried out for 20 min at 25 °C with 5

immunocytochemistry—To stain neurons, the cultured cells were fixed with 4% paraformaldehyde for 20 min and incubated with anti-

Immunoblotting—Cells were lysed in SDS lysis buffer containing 1% SDS, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 8.0), 10 mM NaF, 2 mM Na₃VO₄, 0.5 mM phenylarsine oxide, 10 mM Na₄P₂O₄, and 1 mM phenylmethylsulfonyl fluoride. The lysates were boiled for 3 min and then clarified by ultracentrifugation.

immunoblotting were probed with 1:200 anti-phospho-JNK antibody, 1:200 anti-JNK antibody, 1:200 anti-phospho-p38 antibody, 1:1000 anti-phospho-c-Jun antibody, or 1:1000 anti-c-Jun antibody and then alkylated of 10 µg of protein were resolved by electrophoresis on 10% SDS-polyacrylamide gels. Proteins were transferred onto polyvinylidene fluoride membranes (Millipore Corp.) in 0.1 M Tris base, 0.192 mM glycine, and 20% methanol using a semi-dry electrophoretic transfer system. The membranes were blocked with 0.1% Tween-20 in Tris-buffered saline (T-TBS) containing 5% nonfat dried milk at room temperature for 1 h. Membranes were washed at least 4 times with T-TBS and then incubated with the antigen at 4 °C for 1 h. The membranes were washed at least 4 times with T-TBS and were visualized using the ECL chemiluminescence kit (Amersham Pharmacia Biotech) or Immunostar (Wako).

In Vitro Kinase Assay—The cell lysates were prepared at 0, 3, 6, and 9 h after changing to LK medium using Triton lysis buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA (pH 8.0), 10 mM NaF, 2 mM Na₃VO₄, 0.5 mM phenylarsine oxide, 10 mM Na₄P₂O₄, 2 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Then 1 µg of anti-phospho antibody was added to the lysates followed by incubation at 4 °C for at least 3 h. Protein G-Sepharose (10-µl gel) was then added and rotated at 4 °C for 1 h. The immune complexes were pelleted by centrifugation at 10,000 × g at 4 °C for 1 min and then washed twice with Triton lysis buffer and twice with kinase buffer containing 40 mM HEPES (pH 7.4), 10 mM MgCl₂, 3 mM MnCl₂, 0.1% Triton X-100, 5% nonfat dried milk, and 10 µCi of [γ-32P]ATP, 20 µM ATP. After SDS-polyacrylamide gel electrophoresis, the incorporation of [32P] into GST-ATF2 (1–109) or GST-c-Jun (1–125) was visualized using a Fuji BAS2000 (Amersham Pharmacia Biotech) and the radioactivity was quantified using a Fuji BAS2000 image analyzer.

RESULTS

LK-induced Apoptosis of Cerebellar Granule Neurons—We utilized primary cultures of cerebellar granule cells from neonatal rats as a model system to investigate the intracellular signaling in LK-induced cell death. This cell death shows characteristic features of apoptosis (14, 15). After maturation for 4 days in HK medium, the medium was switched to serum-free LK medium. At 24 h after reduction of potassium concentration, cells were stained with anti-MAP2 antibody (Fig. 1A), and cell survival was determined by MTT assay (Fig. 1B). As a result, 51.7% of granule neurons died in LK medium.

The Phosphorylation and Expression of c-Jun in LK-induced Cell Death—Previous studies showed that the level of c-Jun expression was increased, and the N-terminal phosphorylation of c-Jun was necessary for the process of apoptosis (11, 19). Therefore, we performed Western blotting analysis with monoclonal anti-phosphorylated c-Jun (Ser-63) antibody to detect the phosphorylation of c-Jun (Fig. 2A). Three hours after changing to LK medium, the level of c-Jun phosphorylation was increased, and this phosphorylation was maintained for 12 h. This phosphorylated and activated c-Jun may form homo- or heterodimers with c-Fos, and the dimerized c-Jun may regulate the expression of c-Jun itself. The c-Jun up-regulation is known to be important in DNA damage-induced apoptosis (20) and apoptosis induced by the PI3-K inhibitor, LY294002, in cerebellar granule cells (21). Therefore, we examined the level of c-Jun protein during LK-induced cell death using a polyclonal anti-c-Jun antibody (Fig. 2B). Our results indicated that the expression of c-Jun was markedly increased from 6 to 12 h after potassium deprivation. The delay in the changes in the protein level compared with the phosphorylation of c-Jun was considered to be due to the period required for transcription and translation of c-Jun. In contrast, the phosphorylation and protein levels of c-Jun in serum-free HK-MEM were not changed from basal level.

p38 Was Activated in LK-induced Apoptosis—Next we investigated what kind of kinase phosphorylates c-Jun in cerebellar granule neurons. The first candidate was c-Jun N-terminal kinase (JNK) that belongs to the MAP kinase superfamily and is activated by phosphorylation on Thr-183 and Tyr-185. JNK activation is known to participate in apoptosis of brain neurons and PC12 cells (22–24). In cerebellar granule neurons, glutamate-induced excitotoxicity and DNA damage activated JNK (7, 25). We examined whether JNK was activated to phosphorylate c-Jun during LK-induced apoptosis. To examine JNK activation, the lysates from granule cells cultured for 0, 3, 6, 9, and 12 h in serum-free LK or HK medium after maturation were immunoblotted with anti-phospho-JNK (Fig. 3A). We could not detect any increase in the JNK phosphorylation after lowering potassium concentration. This result suggested that JNK is not involved in c-Jun phosphorylation during LK-induced apoptosis of granule cells, consistent with the observations of Watson et al. (11).

The next candidate was p38, which also belongs to the MAP kinase superfamily. To examine whether p38 was activated during potassium deprivation-induced apoptosis, lysates from granule cells cultured for 0, 3, 6, 9, and 12 h in serum-free LK or HK medium were immunoblotted with anti-phospho-p38 antibody (Fig. 3B). p38 was markedly phosphorylated within 3 h, and the increased level of phosphorylation was prolonged to 9 h after potassium deprivation. To confirm this observation,
we further performed in vitro kinase assay of p38 (Fig. 3C). p38 was immunoprecipitated from the lysates with anti-p38 antibody, and the p38 kinase assay was carried out with [γ-32P]ATP using GST-ATF2-(1–109) as a substrate. ATF2 was markedly phosphorylated by p38 3 h after potassium deprivation. These results indicated that p38 is indeed activated during LK-induced cell death.

**FIG. 3. p38 activation during LK-induced cell death.** After maturation for 4 days, the lysates were prepared from granule neurons cultured for 0, 3, 6, 9, and 12 h in serum-free LK- or HK-MEM, using SDS lysis buffer. The lysates were immunoblotted with anti-phospho-JNK and anti-JNK antibody (A) or anti-phospho-p38 and anti-p38 antibody (B). C, the cell lysates were prepared at 0, 3, 6, and 9 h using Triton X lysis buffer. p38 MAP kinase was immunoprecipitated with 1 μg of anti-p38 antibody. The kinase reaction was carried out for 20 min at 25 °C with the p38 immunoprecipitate and 5 μg of GST-c-Jun-(1–125) as a substrate in kinase buffer. After SDS-polyacrylamide gel electrophoresis, the 32P incorporated into GST-c-Jun-(1–125) was visualized using a Fuji BAS2000 image analyzer (upper). The immunoprecipitates were immunoblotted with anti-p38 antibody (bottom).

**FIG. 4. Involvement of p38 in c-Jun phosphorylation during LK-induced apoptosis.** A, after maturation for 4 days, the lysates were prepared from granule neurons cultured for 0, 3, 6, and 9 h in serum-free LK- or HK-MEM with or without SB203580 using the SDS lysis buffer. The lysates were subjected to Western blotting analysis using anti-phospho-c-Jun antibody (upper) and anti-c-Jun antibody (bottom). B, the granule neurons were cultured for 0, 3, 6, and 9 h after changing to LK-MEM. The cell lysates were prepared using Triton-X lysis buffer. p38 was immunoprecipitated with 1 μg of anti-p38 antibody. The p38 kinase reaction was carried out for 20 min at 25 °C with the immunoprecipitate and 5 μg GST-c-Jun-(1–125) as a substrate in kinase buffer. After SDS-polyacrylamide gel electrophoresis, the 32P incorporated into GST-c-Jun-(1–125) was visualized using a Fuji BAS2000 image analyzer (upper). The immunoprecipitates were immunoblotted with anti-p38 antibody (bottom).

**Direct Phosphorylation of c-Jun by p38 in Vitro—** It is an intriguing question whether p38 phosphorylates c-Jun. As shown in Figs. 2A and 3B, the time courses of phosphorylation of p38 and c-Jun were very similar. To examine whether p38 is involved in the regulation of c-Jun, we investigated the effects of SB203580, a specific inhibitor of p38, on the phosphorylation and expression of c-Jun. The addition of SB203580 to LK medium markedly suppressed the phosphorylation and up-regulation of c-Jun in a dose-dependent manner (Fig. 4A). Next, we examined whether p38 directly phosphorylated c-Jun using in vitro kinase assay. After immunoprecipitation with anti-p38 antibody, the p38 kinase assay was carried out with [γ-32P]ATP, using GST-c-Jun-(1–125) as the substrate. At 3 h after potassium deprivation, c-Jun was phosphorylated by p38 (Fig. 4B). These results suggested that p38 directly phosphorylates c-Jun during LK-induced apoptosis of granule cells.

**SB203580 Prevented LK-induced Apoptosis—** To examine whether the activation of p38 is involved in the process of LK-induced apoptosis, we utilized SB203580, an inhibitor of p38. When apoptosis was induced by lowering the potassium concentration, the inhibitor was added to LK medium (Fig. 5). After 24 h, cell survival was quantified by counting the number of MAP2-positive cells. Resultingly SB203580 inhibited neuronal apoptosis. In the presence of 3, 10, and 30 μM SB203580, 59, 74, and 81% of cells survived, respectively. This result indicated that p38 is involved in the signaling pathway of LK-induced apoptosis.

**DISCUSSION**

Several recent studies have suggested that p38 is required for some apoptotic processes (5, 6). For example, during glutamate-evoked apoptosis of cerebellar granule neurons, p38 was transiently activated, and this apoptosis was prevented by SB203580 (7). However, function of p38 in apoptotic process remains unclear. In this study, we investigated the role of p38 in LK-induced apoptosis of cultured cerebellar granule neurons. Our results indicated that p38 was phosphorylated and activated during LK-induced apoptosis (Fig. 3, B and C). In Fig.
3B, control neurons in HK medium also showed a transient and slight activation of p38. The transient activation of p38 was considered to be due to changing from serum-containing to serum-free HK-MEM.

Brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-1 (IGF-1) are known to rescue granule neurons in cerebellar granule neurons, Watson et al. (11) showed that dominant negative c-Jun Ala, which cannot be phosphorylated, completely prevented both phosphorylation and up-regulation of c-Jun. It has been reported that SB203580, which is a specific inhibitor of p38, can directly phosphorylate c-Jun during LK-induced apoptosis (Fig. 4B). The phosphorylation of c-Jun was confirmed by SB203580 (data not shown).

The mRNA and protein levels of c-Jun were selectively increased after NGF withdrawal in sympathetic neurons (10, 28). In cerebellar granule neurons, Watson et al. (11) showed that dominant negative c-Jun Ala, which cannot be phosphorylated, inhibited LK-induced apoptosis. We observed marked changes in the levels of phosphorylation and expression of c-Jun during this apoptotic process. Although the importance of c-Jun is well known, its kinase has remained unclear. The first candidate was JNK, because the phosphorylation of c-Jun by JNK was well known in sympathetic neurons (29). However, in cerebellar granule neurons, the JNK activity was high under basal conditions and was not activated further during apoptosis. The next candidate was p38, because the time courses of the phosphorylation of p38 and c-Jun during apoptosis were very similar. To investigate the role of p38 in the phosphorylation of c-Jun, we utilized SB203580. Further, we found that SB203580 prevented LK-induced apoptosis in a dose-dependent manner (Fig. 5). This prevention of apoptosis seemed to be due to the inhibition of phosphorylation of c-Jun. It has been reported that SB203580 can block some JNKs at high doses (30–32). Since we could not detect low potassium-induction of JNKs, its contribution to the present findings is likely to be limited but warrants further investigation. SB202190 is also utilized as a specific inhibitor of p38 (33). We observed a weak but significant inhibitory effect of SB202190 on the apoptosis (data not shown).

ATF2 is a member of the ATF/CREB family of basic region leucine zipper (bZIP) DNA-binding proteins. The N-terminal transactivation domain of ATF2 is phosphorylated by both p38 and JNK (34–36). Therefore, we investigated whether ATF2 was also phosphorylated by p38 during LK-induced apoptosis. The phosphorylation of ATF2 was detected by Western blotting analysis using anti-phospho-ATF2 antibody. ATF2 was markedly phosphorylated at 3 h (data not shown). The time course of ATF2 phosphorylation corresponded to those of phosphorylation of p38 and c-Jun. These observations suggested that p38 is involved in phosphorylation of not only c-Jun but also of ATF2. We observed that p38 showed greater affinity for ATF2 than c-Jun as a substrate in the kinase assay utilizing the same amounts of GST-ATF2 and GST-c-Jun (data not shown). In addition, we investigated whether SB203580 inhibited phosphorylation of ATF2 as well as that of c-Jun. SB203580 indeed inhibited ATF2 phosphorylation (data not shown).

The results presented here demonstrate that p38 can directly phosphorylate c-Jun during LK-induced apoptosis in cultured cerebellar granule neurons. However, the mechanisms of c-Jun-mediated apoptosis remain unclear. Therefore, further studies should be performed to identify c-jun target genes and to determine how c-Jun regulates apoptosis.

Acknowledgments—We thank Regeneron Pharmaceutical Co. for the kind gifts of BDNF; Dr. H. Murofushi (The University of Tokyo) for anti-MAP2 antibody; and Dr. N. Nomura (Razusu DNA Research Institute) for c-Jun DNA.

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