An Inhibitor of p38 and JNK MAP Kinases Prevents Activation of Caspase and Apoptosis of Cultured Cerebellar Granule Neurons

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Received November 30, 1998 Accepted December 25, 1998

ABSTRACT—Both p38 mitogen-activated protein kinase (p38) and c-Jun N-terminal kinase (JNK) are known to play important roles in neuronal apoptosis. However, the relationship between these kinases and caspases, another key mediator of apoptosis, is unclear. In the present study, we investigated the possible effects of SB203580 [(4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole], an inhibitor of p38, on caspase activation and apoptosis of cultured rat cerebellar granule neurons. In granule neurons, SB203580 prevented apoptosis that was induced by lowering the concentration of KCl in the culture medium for 24 hr. SB203580 also prevented augmentation of caspase-3-like protease activity at 8 hr after the low KCl treatment. The IC_{50} values of SB203580 for both events were between 3 μ M and 10 μ M. Expression and phosphorylation of c-Jun, potently induced by low KCl treatment, were prevented by SB203580 at 10 μ M. Z-Asp-CH₂-DCB, a caspase inhibitor with anti-apoptotic activity, did not inhibit the induction and phosphorylation of c-Jun. Granule neurons displayed high levels of p38 and JNK activities. SB203580 inhibited not only p38 but also JNK activities extracted from granule neurons. These results suggest that activation of c-Jun by p38 and/or JNK mediates the activation of caspase in the low KCl-induced apoptosis in cerebellar granule neurons.

Keywords: Apoptosis, p38, Jun N-terminal kinase, Caspase-3, Cerebellar granule cell

The mitogen-activated protein (MAP) kinase family plays a pivotal role in the response of cells to extracellular stimuli (1–3). There are at least three distinct MAP kinases: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), which are also called stress-activated protein kinases (SAPKs), and the p38 MAP kinases (p38s, also called CSBP/RK/MPK2). Recent studies have suggested that JNK and p38 play critical roles in neuronal apoptosis. In neuronally differentiated PC12 cells, Xia et al. showed that using dominant-negative or constitutively active forms of various components of the ERK-, JNK- and p38-signaling pathways, either activation of the ERK-signaling pathway or inhibition of either the JNK or the p38 pathway is sufficient to block apoptosis that was induced by nerve growth factor (NGF) withdrawal (4). Activation of c-Jun transcription factor, which is mediated by phosphorylation at Ser 63/73 by JNK, has been shown to be necessary for apoptosis of superior cervical ganglion (SCG) neurons after NGF withdrawal (5, 6).

In brain neurons, involvement of JNK (7–9) and p38 (10, 11) in apoptosis has also been reported. In cerebellar

granule neurons, for example, JNK and p38 are activated before glutamate-induced apoptosis of cultured cerebellar granule neurons (11). SB203580 [(4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole] (Fig. 1), an inhibitor of p38 (12, 13), prevented the glutamate-induced apoptosis (11). Watson et al. reported that c-Jun was induced and phosphorylated in granule neurons before apoptosis was induced by lowering the KCl concentration in the medium and that the expression of dominant-negative mutant of *c-jun* prevented apoptosis (9).

Caspases also play a pivotal role in the apoptosis of brain neurons (14, 15). We (16, 17) and others (18–20) have demonstrated that the activation of caspase-3 or closely related caspase(s) is essential for low KCl-induced apoptosis of cerebellar granule neurons. Thus, both the “stress-activated” protein kinases, including JNK/p38, and the caspase(s) are thought to be necessary to cause apoptosis of granule neurons. However, the relationship between these two pathways in apoptosis of neurons is not fully understood. In apoptosis of PC12 cells (21) and SCG neurons (22) induced by NGF withdrawal, it has

Fig. 1. Chemical structures of SB203580 (upper), SB202190 (middle) and SB202474 (lower).

been suggested that activation of JNK is either independent of or lies upstream of the activation of caspase. There are controversial results about the relationship between JNK/p38 and the caspases in several models of apoptosis of non-neuronal cells. In TNF-induced apoptosis, activation of JNK (23, 24) and p38 (25) is dispensable for apoptosis and possibly independent of activation of the caspase. However, ASK1, an activator of JNK/p38 signaling, is shown to be necessary for TNF-induced apoptosis (26). Fas-induced activation of JNK (27) and p38 (28) requires caspase activity. However, the activation of JNK and p38 is independent of both Fas-induced activation of caspase-3 and apoptosis (28–31).

To investigate the possible relationship between p38 and caspase in the low KCl-induced apoptosis of granule neurons, we examined the effect of SB203580 on low KCl-induced activation of caspase and apoptosis. In the course of experiments, we unexpectedly found that SB203580 inhibited low KCl-induced phosphorylation of c-Jun, possibly due to its inhibitory effect on JNK as well as on p38 in granule neurons. Our results suggest that activation of c-Jun, which is mediated by p38 and/or JNK, is required for the activation of caspase during the apoptosis of granule neurons.

MATERIALS AND METHODS

Drugs

SB203580 (Fig. 1) was synthesized in-house as well as purchased from Calbiochem (La Jolla, CA, USA). Both preparations gave similar results. SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-imidazole] and SB202474 [4-ethyl-2-(4-methoxyphenyl)-5-(4-pyridyl)-imidazole] (Fig. 1) were also purchased from Calbiochem. Actinomycin D was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Benzylloxycarbonyl-Asp-CH₂-DCB (Z-Asp) was purchased from Funakoshi Co. (Tokyo).

Preparation of cultured cerebellar granule neurons and treatment of cultures

Primary cultures of cerebellar granule neurons were obtained from the cerebella of 7- to 8-day-old Sprague-Dawley rats (Japan SLC, Hamamatsu), as described previously (16). In brief, the cerebella were isolated and then dissected and trypsinized at 37°C. The dissociated cells were seeded onto 48-well culture plates precoated with poly-D-lysine at a density of $3-4 \times 10^5$ cells/cm² in basal modified Eagle's medium containing 25 mM KCl, 10% FBS, 2 mM glutamine and 50 µg/ml gentamicin. Cytosine arabinofuranoside (10 µM) was added 18–24 hr after plating to inhibit the growth of non-neuronal cells. Five to six days after plating, the culture medium was removed, washed once and replaced with a serum-free minimum essential medium (MEM) containing 5.6 mM KCl (low KCl) with or without drugs such as SB203580. Some wells in each plate were washed and replaced with a serum-free medium containing 25 mM KCl (high KCl), and some wells were left alone without medium change (intact cells).

Morphological examination of viable and dead neurons

Double staining of both viable and dead neurons (17) was used. Cells were incubated at 37°C for 30 min with MEM containing 1 µM calcein acetoxymethyl ester (calcein), which is cleaved by esterases present in living cells, yielding yellowish-green fluorescence, and 10 µg/ml propidium iodide (PI), which is taken up in dead cells and becomes orange-red fluorescent by intercalating into DNA. Both living and dead cells can be simultaneously observed in a Diaphot-TMD fluorescence microscope (Nikon, Tokyo).

Measurement of LDH activity

Neurons were treated for 24 hr in triplicate wells as described above. Lactate dehydrogenase (LDH) activity released from dead cells was determined with a kit (Boehringer Mannheim, Mannheim, Germany) as

described previously (16). The absorbance (Abs) at 490 nm was determined with a microplate reader. The prevention of low KCl-induced LDH release by the drugs was expressed as follows:

$$\{[\text{Abs}(\text{low KCl plus drugs}) - \text{Abs}(\text{low KCl})] / [\text{Abs}(\text{high KCl}) - \text{Abs}(\text{low KCl})]\} \times 100 (\%)$$

Detection of caspase-3-like proteolytic activity

Neurons were treated for 8 hr in triplicate wells as described. Then, the culture medium was removed, and the cells were washed once with phosphate-buffered saline (PBS) and lysed in a hypotonic reaction buffer [20 mM Hepes-NaOH (pH 7.5), 2 mM dithiothreitol (DTT)] containing 20 μ M of a fluorogenic substrate, Acetyl-Asp-Glu-Val-Asp-4-methylcoumaryl-7-amide (Ac-DEVD-MCA). After the reaction mixtures were incubated at 37°C for 1 hr, fluorescence was measured using a Cytofluor 2350 plate reader, at an excitation wavelength of 360 nm and an emission wavelength of 460 nm, and expressed as change in fluorescence units (FU). Prevention of low KCl-induced caspase-3 activation by the drugs was expressed as follows:

$$\{1 - [\text{FU}(\text{low KCl plus drugs}) - \text{FU}(\text{intact cells})] / [\text{FU}(\text{low KCl}) - \text{FU}(\text{intact cells})]\} \times 100 (\%)$$

Western blotting

Granule neurons were grown in 6-well plates and treated as described above. At indicated times after treatment, culture medium was removed, and cells were lysed in 200 μ l of Laemmli sample buffer. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Membranes were probed with respective antibodies according to the instructions of the manufacturer (New England BioLabs, Beverly, MA, USA). The antibodies used were rabbit polyclonal anti-p38 MAPK antibody, rabbit polyclonal phospho-specific p38 MAPK (Thr180/Tyr182) antibody; rabbit polyclonal anti-SAPK/JNK antibody, rabbit polyclonal phospho-specific SAPK/JNK (Thr183/Tyr185) antibody; rabbit polyclonal anti-activating transcription factor-2 (ATF-2) antibody, rabbit polyclonal phospho-specific ATF-2 (Thr71) antibody; rabbit polyclonal anti-c-Jun antibody, and rabbit polyclonal phospho-specific c-Jun (Ser73) antibody.

Determination of p38 and JNK activities

P38 activity was measured by immune complex kinase assay and JNK activity was measured by solid phase kinase assay using nonradioactive assay kits (New England BioLabs). In brief, the cells were washed with PBS and extracted in ice-cold lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -

glycerophosphate, 1 mM Na_3VO_4 , 1 μ g/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride]. For the p38 assay, cell lysates were incubated overnight with rabbit anti-p38 antibody and then incubated with protein A-agarose beads. The beads were washed twice with lysis buffer and twice with kinase buffer [25 mM Tris (pH 7.5), 5 mM β -glycerophosphate, 0.1 mM Na_3VO_4 , 2 mM DTT and 10 mM MgCl_2]. The kinase reaction was started by the addition of 2 μ g of glutathione *S*-transferase (GST)-ATF-2 (19–96) and 200 μ M ATP and terminated by the addition of Laemmli sample buffer after incubation for 30 min at 30°C. In some experiments, SB203580 was added 20 min prior to the start of reaction. Phosphorylated GST-ATF-2 was detected by Western blotting as described above. For JNK assay, JNK was pulled down from cell lysates by incubation with GST-c-Jun (1–89) beads, washed as above and incubated with 100 μ M ATP. Phosphorylated GST-c-Jun was detected as described above. The autoradiograms were analyzed using the Kodak digital science electrophoresis documentation and analysis system, and the intensity of respective bands was calculated using 1D image analysis software (Kodak, Rochester, NY, USA).

RESULTS

Effects of SB203580 on low KCl-induced apoptosis

Cerebellar granule neurons grown in the medium containing 25 mM KCl (high KCl) underwent apoptosis when the medium was changed to that containing 5.6 mM KCl (low KCl treatment) as reported previously (16–20). When SB203580 was added to the low KCl medium, it prevented the apoptosis normally occurring at 24 hr after the treatment. Figure 2 shows results of morphological examination using calcein/PI staining. When the neurons were treated with low KCl medium for 24 hr, many neurons stained red by PI, indicating extensive neuronal death (Fig. 2B). In contrast, most neurons were living after high KCl treatment (Fig. 2C) as well as neurons without treatment (Fig. 2A). SB203580 at 10 μ M prevented the low KCl-induced neuronal death (Fig. 2D). Effect of SB203580 on neuronal death was concentration-dependent, as determined by LDH release from dead neurons (Fig. 3). The IC_{50} of SB203580 for LDH release was $9.0 \pm 1.8 \mu\text{M}$ (mean $\pm$ S.E.M. of 4 independent experiments). SB202190 (Fig. 1), a compound structurally similar to SB203580 and which inhibits p38 (12), was also as effective as SB203580 in preventing low KCl-induced neuronal death at 10 μ M. On the other hand, SB202474 (Fig. 1), a compound that lacks the inhibitory effect on p38 (12), was ineffective on the neuronal death up to 30 μ M (data not shown).

Fig. 2. Effects of SB203580 on low KCl-induced apoptosis of cerebellar granule neurons determined by calcein/PI staining. Neurons were treated with medium containing low KCl (B), high KCl (C), or low KCl plus 10 μ M SB203580 (D) or left without treatment (A). Twenty-four hours later, living cells were stained green by calcein and dead cells were stained red by PI. Bar = 50 μ m.

Fig. 3. Preventive effect of SB203580 on low KCl-induced apoptosis determined by LDH release. Cerebellar granule cells were treated with MEM containing 5.6 mM KCl and the indicated concentrations of SB203580. LDH release in the culture medium was determined at 24 hr after treatment. Prevention of low KCl-induced LDH release by SB203580 was calculated and expressed as described in Materials and Methods. LDH release from cells treated with low KCl and high KCl was $11.4 \pm 2.7\%$ and $3.7 \pm 0.3\%$, respectively, of total cellular LDH activity. Data represent the mean $\pm$ S.E.M. of 4 independent experiments.

Fig. 4. Preventive effect of SB203580 on low KCl-induced activation of caspase-3-like protease. Cerebellar granule cells were treated with MEM containing 5.6 mM KCl and indicated concentrations of SB203580. Caspase-3-like Ac-DEVD-MCA cleavage activity was determined at 8 hr after treatment. Prevention of low KCl-induced activation of caspase by SB203580 was calculated and expressed as described in Materials and Methods. Ac-DEVD-MCA cleavage activity in cells treated with low KCl and high KCl was $404 \pm 128\%$ and $94 \pm 6\%$, respectively, of the activity in intact cells. Data represent the mean $\pm$ S.E.M. of 3 independent experiments.

Fig. 5. Time course of low KCl-induced changes in expression- and phosphorylation patterns of stress-related proteins. Cerebellar granule cells were treated with MEM containing 5.6 mM KCl (low KCl) or 25 mM KCl (high KCl) for the indicated time. Cellular proteins were extracted and separated by SDS-PAGE and probed with respective antibodies. The asterisk marks a nonspecific band detected by the anti-c-Jun antibody. The data shown are representative of 2 independent experiments.

Effects of SB203580 on low KCl-induced activation of caspase-3-like protease

In the low KCl-induced apoptosis of granule neurons, caspase-3-like protease is activated preceding the apoptosis, as determined by cleavage of synthetic substrate Ac-DEVD-MCA (16–20). Activation of the caspase-3-like activity, determined at 8 hr after the low KCl treatment, was suppressed by SB203580 with a concentration dependency similar to its effect on neuronal death (Fig. 4). The IC_{50} of SB203580 for activation of caspase was $3.3 \pm 0.4 \mu\text{M}$ (mean $\pm$ S.E.M. of 3 independent experiments). As well as its effect on neuronal death, SB202190 (10 μM), but not SB202474 (30 μM), prevented the low KCl-induced the activation of caspase (data not shown).

Expression and phosphorylation of p38, JNK and their substrates in granule neurons

Phosphorylation status of MAP kinases, as well as that of their substrates, usually reflects their activities. In the

next series of experiments, expression and phosphorylation of p38, JNK, ATF-2 (a substrate of p38 and JNK) and c-Jun (a substrate of JNK) after the low KCl treatment were examined by Western blotting using respective antibodies (Fig. 5). p38, JNK (2 species of p54 and p46) and ATF-2 were constitutively expressed in the granule neurons. Substantial amounts of these proteins were phosphorylated in the untreated control cells. Expression and phosphorylation of p38 and JNK were affected little by either low KCl treatment or high KCl treatment. In contrast, phosphorylation of c-Jun at Ser 73 was remarkably induced as early as 1 hr after low KCl treatment, similar to the phosphorylation at Ser 63 (9). Expression of c-Jun was also augmented from 2 hr after low KCl treatment. High KCl treatment did not induce these changes on c-Jun. Phosphorylation of ATF-2 was slightly, but reproducibly, increased by the low KCl treatment but not by high KCl treatment.

Fig. 6. Effect of drugs on low KCl-induced changes in expression- and phosphorylation patterns of stress-related proteins. Cerebellar granule cells were treated with MEM containing 25 mM KCl (high KCl) or 5.6 mM KCl (low KCl) in the absence or presence of the indicated drugs for 4 hr. Drugs used were Z-Asp-CH₂-DCB (Z-Asp, 100 μM), actinomycin D (1 $\mu\text{g}/\text{ml}$), SB203580 (10 μM or 30 μM). Cellular proteins were extracted and separated by SDS-PAGE and probed with respective antibodies. The data shown are representative of 3 independent experiments.

Effects of SB203580 on low KCl-induced changes of p38 and JNK substrates

Figure 6 shows the effect of SB203580 on expression and phosphorylation of p38, JNK, ATF-2 and c-Jun

Fig. 7. p38 and JNK activities in granule neurons and effect of SB203580 in vitro. **A:** Cerebellar granule cells were treated with MEM containing 5.6 mM KCl (low KCl) or 25 mM KCl (high KCl) for 2 hr. Kinase activities in cellular extracts were assayed as described in Materials and Methods. The data shown are representative of 3 independent experiments. **B:** Cerebellar granule cells were treated with MEM containing 5.6 mM KCl for 2 hr. SB203580 at indicated concentrations was added to aliquots of sample prior to the start of kinase reactions. The autoradiograms of one typical experiment are shown in the upper panel. The graph shows relative activities of p38 (●) or JNK (○) compared to the activities of no-drug control. Data represent the mean $\pm$ S.E.M. of 3 independent experiments.

measured at 4 hr after the low KCl treatment. Effects of Z-Asp (a nonselective inhibitor of caspases) and actinomycin D (an inhibitor of RNA synthesis), potent anti-apoptotic drugs on granule neurons (9, 17, 18), were also examined. SB203580 showed little effect on the phosphorylation of p38 and JNK. In contrast, SB203580 potently suppressed the increased levels of expression and phosphorylation of c-Jun by low KCl treatment at 10 μ M and 30 μ M. Phosphorylation of ATF-2 was also reduced by SB203580 at 30 μ M. Actinomycin D potently inhibited the stimulated expression of c-Jun, while it only weakly inhibited the phosphorylation of c-Jun. Z-Asp affected neither the induction nor the phosphorylation of c-Jun. The preventive effects of SB203580 on the induction and phosphorylation of c-Jun were also observed in the samples treated with low KCl for 8 hr (data not shown).

p38 and JNK activities in granule neurons

The in vitro kinase was performed to ascertain whether constitutive phosphorylation of p38 and JNK actually reflects their activity (Fig. 7). Consistent with their phosphorylation status, the granule neurons possessed high levels of p38 and JNK activities. These activities did not change 2 hr after low KCl treatment (Fig. 7A). Both p38 and JNK activities in the granule neurons were several times higher than those in PC12 cells that were treated with anisomycin (data not shown). The inhibitory effect of SB203580 on p38 and JNK activities that were extracted from granule neurons were examined by in vitro kinase assays (Fig. 7B). Activity of p38 extracted from granule neurons was inhibited by SB203580 at concentrations that prevented both caspase activation and apoptosis in cells. In addition, the JNK activity extracted from granule neurons was also inhibited by similar concentrations of SB203580 (Fig. 7B).

DISCUSSION

In the present study, similar concentrations of SB203580 inhibited both the low KCl-induced apoptosis (Figs. 2 and 3) and the activation of caspase-3-like protease (Fig. 4). These results suggest that SB203580 prevented apoptosis of granule neurons by suppressing the activation of caspase, similar to other agents that prevent apoptosis in this model (17). Addition of SB203580 to the reaction buffer to measure caspase activity did not inhibit its activity up to 100 μ M (data not shown), suggesting that the molecular target of SB203580 is not caspase itself but is upstream of the activation of caspase. Furthermore, SB203580 inhibited low KCl-induced expression and phosphorylation of c-Jun at the same concentrations at which it blocked apoptosis (Fig. 6). To our knowledge, this is the first observation that

SB203580 inhibited phosphorylation of c-Jun in cultured cells. Activation of c-Jun is considered a key event in several models of neuronal death (5–9, 32, 33). Actually, the low KCl-induced apoptosis of cerebellar granule neurons was prevented by expression of a dominant-negative mutant of *c-jun* (9). Thus, it is likely that an inhibition of c-Jun contributes to the anti-apoptotic effect of SB203580. Although Z-Asp, a caspase inhibitor, prevents low KCl-induced apoptosis (17, 19), it did not affect either the induction or the phosphorylation of c-Jun (Fig. 6). Similarly, in NGF-deprived PC12 cells (21) and SCG neurons (22), caspase inhibitors did not inhibit the activation of JNK or the induction of *c-jun* but they prevented apoptosis. Phosphorylation of c-Jun increased as early as 1 hr after the low KCl treatment (Fig. 5), and it apparently preceded the activation of caspase-3-like protease, which was not detected until 3–4 hr after the low KCl treatment (17–19). Thus, activation of c-Jun and of caspase-3-like protease probably occurs sequentially in neuronal apoptosis.

Actinomycin D was mostly ineffective on the low KCl-induced phosphorylation of c-Jun (Fig. 6). However, it potently inhibited the induction of c-Jun (Fig. 6) and caspase activation (17). Actinomycin D probably prevented apoptosis by blocking the induction of some gene products that are involved in caspase activation, and c-Jun may regulate the transcription of these gene products.

SB203580 has been postulated as a specific inhibitor of p38. However, recent reports (34, 35) have suggested that SB203580 also inhibits particular isoforms of JNK (e.g., IC_{50} value is less than 10 μ M for JNK2 β). In the present study, half of the JNK activity extracted from granule neurons was inhibited by 10 μ M SB203580 (Fig. 7B). Thus, inhibition of JNK by SB203580 probably contributes to the inhibition of low KCl-induced phosphorylation of c-Jun. Transcription of the *c-jun* gene is regulated by a transcriptional activator AP-1 composed of c-Jun and ATF-2 (36). Although c-Jun is activated by JNK but not by p38, ATF-2 is activated by both JNK and p38. In addition, another transcription factor, MEF2C, which also regulates induction of *c-jun*, is activated by p38 (37). Thus, it is possible that SB203580 suppressed the induction of c-Jun by inhibiting p38 (38), as well as by inhibiting JNK. The inhibitory effect of SB203580 on p38 activity extracted from granule neurons (Fig. 7B) was weaker than its effect on human recombinant p38 (IC_{50} is less than 1 μ M, ref. 13 and our unpublished observation). Recent reports have shown the existence of p38 subtypes that differ from each other in sensitivity to SB203580 (39–44). The subtype(s) of p38 expressed in the cultured granule neurons remains to be determined.

Both p38 and JNK are constitutively expressed and

phosphorylated (Fig. 5) in active forms (Fig. 7A) in cultured cerebellar granule neurons. These results are similar to those in the previous reports (9, 11), although the basal levels of p38 activity differ in each study. The difference in the levels of p38 activity may be due to the different p38 antibodies used to immunoprecipitate samples. A recent report also demonstrated that JNK is constitutively active in the mouse brain (45). Constitutive activities of p38 and JNK in granule neurons cultured in high KCl medium are possibly maintained by Ca^{2+} /calmodulin-dependent protein kinases (CaM kinases, ref. 46). Consistent with this possibility, activity-dependent survival of cerebellar granule neurons under high KCl medium is maintained by CaM kinase activity (47). Furthermore, the cAMP response element-binding protein (CREB), another substrate of CaM kinases in neurons (48), is constitutively phosphorylated at Ser 133 in granule neurons cultured under high KCl, but not under low KCl medium (J. Harada, unpublished observation). Pyk2, a tyrosine kinase that is activated by stimuli that elevate intracellular calcium in neurons, may also contribute to the activation of p38 and JNK (49, 50).

The lack of apparent changes in p38 and JNK activities after low KCl treatment is contradictory to the drastic induction and phosphorylation of c-Jun after low KCl treatment. A novel mechanism(s) regulating phosphorylated states of c-Jun independently of the activity of p38 and JNK may exist in neurons. It is noteworthy that regulatory mechanisms of MAP kinase cascades, such as association of MAP kinase with phosphatase (51) and complex formation with a scaffold protein (52), were identified recently. The low KCl-induced phosphorylation of c-Jun may be mediated by changes of such regulatory mechanism(s) of MAP kinase cascades.

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