Apoptosis-associated Tyrosine Kinase (AATYK) Has Differential Ca\(^{2+}\)-dependent Phosphorylation States in Response to Survival and Apoptotic Conditions in Cerebellar Granule Cells*

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In dissociated cultures of cerebellar granule cells, extracellular high potassium (HK) and low potassium (LK) concentrations control cell survival and apoptosis, respectively. Apoptosis-associated tyrosine kinase (AATYK) is up-regulated during the LK-induced apoptosis. Overexpression of wild-type AATYK, but not its kinase-deficient mutant, stimulates apoptosis in LK. In this study, we analyzed the relationship between the phosphorylation states of AATYK and the survival of granule cells. AATYK was hypophosphorylated in HK, whereas it was hyperphosphorylated in apoptotic LK. HK-dependent hypophosphorylation of AATYK was controlled by L-type voltage-dependent calcium channel-mediated Ca\(^{2+}\) influx followed by Ca\(^{2+}\)-dependent protein phosphatase activity. However, LK-induced hyperphosphorylation of AATYK at multiple sites was blocked by kainate, lithium, and protein kinase C-\(\delta\) inhibitor. AATYK phosphorylation was concurrent with c-Jun phosphorylation. In addition, mutations of AATYK on either the kinase domain or Ser-480, Ser-558, and Ser-566 residues suppressed the LK-induced hyperphosphorylation and apoptosis, suggesting the involvement of self-kinase activity and these Ser residues in this process. Our data therefore indicate that the phosphorylation states of AATYK are closely related to the HK-induced survival and LK-induced apoptosis of cerebellar granule cells.

Apoptosis is observed not only in neurodegenerative diseases, but also in the normal development of the nervous system. Neurons that are supplied with survival factors (e.g. neurotrophic factors), or synaptic activity through their proper synaptic connections mature and survive during neuronal development. Neurons without synaptic connections are eliminated by apoptosis or undergo programmed cell death because of the absence of these survival factors (1, 2).

Cerebellar granule cells (CGC\(^{2+}\)) prepared from early postnatal mice differentiate into mature neurons and survive for a long time under depolarizing culture conditions with high K\(^{+}\) (HK; 25–30 mM) (3–5). On the other hand, lowering the concentration of K\(^{+}\) to 5 mM (LK) triggers CGC apoptosis (6). This CGC apoptosis is thought to reflect the regulations of the trophic action of neuronal activity and has been intensively used as a model for studying the mechanisms of survival factor withdrawal-induced neuronal apoptosis. A distinctive feature of CGC apoptosis is the requirement of de novo RNA synthesis (6–10). A key transcription factor for apoptosis is c-Jun, an immediate-early gene (10). Deprivation of the survival signal leads to an increase in both the protein level and the N-terminal phosphorylation of c-Jun, and the expression of dominant negative c-Jun inhibits cell death after withdrawal of the survival signals (9, 11–13). On the other hand, serine-threonine protein kinase Akt plays an important role in neuronal survival, and it is activated by the phosphatidylinositol 3-kinase pathway (14, 15) and the Ca\(^{2+}\)-calmodulin-dependent protein kinase pathway (16). Addressing the question of how these signaling molecules behave in both survival and apoptotic conditions will lead to a better understanding of the two opposing cellular mechanisms underlying the survival versus apoptosis of CGCs.

Apoptosis-associated tyrosine kinase (AATYK) was found to be up-regulated during the apoptosis of myeloid precursor cells induced by IL-3 deprivation (17). We showed that AATYK is enriched in mouse brains and was also up-regulated during CGC apoptosis induced by low K\(^{+}\) concentrations (18). It is notable that the molecular mass of the AATYK protein on SDS-PAGE showed a slight increase at 3 h after switching to LK, although the protein expression level was only slightly increased compared with the rapid increase in mRNA level. We previously reported that AATYK is tyrosine-phosphorylated and has in vitro kinase activity (18). Recently, it has been shown that the AATYK family also possesses Ser/Thr kinase activity (19, 20). Treatment with protein tyrosine phosphatase partly reduced this LK-induced molecular shift (18). In addition, the number of apoptotic CGCs overexpressing exogenous AATYK in LK was higher than that of CGCs overexpressing the kinase domain mutant that lacks the self-kinase activity. However, little is known about the molecular mechanism underlying AATYK-associated CGC apoptosis. On the other hand, AATYK promotes the neurite elongation of young CGCs, suggesting that AATYK has dual functions dependent on its developmental stage (21).

To clarify the AATYK-associated CGC apoptosis, we investigated the phosphorylation of AATYK under survival (HK) and apoptotic (LK) conditions. We found that AATYK is hypophosphorylated in HK but is hyperphosphorylated in LK. The HK-induced hypophosphorylation state depends on the depolarization-induced Ca\(^{2+}\) signaling pathway, whereas the LK-induced hyperphosphorylation state is partly attributed

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2 The abbreviations used are: CGC, cerebellar granule cell; AATYK, apoptosis-associated tyrosine kinase; VDCC, voltage-dependent \(Ca^{2+}\) channel; BAPTA-AM, 1,2-bis(2-aminoophenoxy)ethane-N,N,N,N-tetraacetic acid acetoxymethyl ester; Z-VAD-FMK, N-benzoyloxycarbonyl-Val-Ala-Asp(Ome)-fluoromethyl ketone; AMPA, \(\alpha\)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; NMDA, \(N\)-methyl-D-aspartate acid; DNXQ, 6,7-dinitroquinoxaline-2,3(1H,4H)-dione; NBQX, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxolo-benzof[1,2-c:4,5-c]quinoline-5-sulfonamide; DAPI, 4',6-diamidino-2-phenylindole; APV, D(-)-2-amino-5-phosphono pentanoic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; JNK, c-Jun N-ter-
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to self-kinase activity. This study suggests that the switching between these two phosphorylation states regulates CGC survival and apoptosis.

MATERIALS AND METHODS

Chemicals—The chemicals were purchased from the following sources. SB203580, PD98059, LY294002, Z-VAD, roscovitine, BAPTA-AM, nimodipine, FK-506, A23187, (−)-BayK8644, rotllerin, staurosporin, herbimycin A, and genistein were from Calbiochem (La Jolla, CA); nifedipine, AMPA, NMDA, DNQX, NBQX, and APV were from Tocris Cookson (Ballwin, MO). Kainic acid, thapsigargin, N-acetyl cysteine, insulin-like growth factor 1 (IGF-1), lithium chloride, 2-propylpentanoic acid, sodium salt (sodium valproic acid), rotllerin, and oryroniositol were from Sigma. KN62 was from Seikagaku-kogyo (Tokyo, Japan). Cyclosporin A and α-conotoxin were from Wako Pure Chemicals Inc. (Tokyo, Japan).

Cell Culture—Primary cultures of CGCs were prepared from mice on postnatal day 7 as described previously (22). Briefly, CGCs were seeded at a density of 2.5 × 10⁵ cells/cm² and grown on poly-L-lysine-coated plates in an HK medium, neurobasal medium containing B27 supplement (Invitrogen), 25 mM KCl, 1 mM glutamine, penicillin (50 units/ml), and streptomycin (50 μg/ml). To induce apoptosis, the medium was replaced with fresh medium containing 5 mM KCl (LK) and B27 after 7 days of culture in HK.

Measurement of Cell Viability—The mitochondrial dehydrogenase activity that reduces 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in active mitochondria to purple formazan was used to determine cell survival. The cells were incubated with MTT (0.2 mg/ml) for 60 min, then washed with lysis buffer (50% N,N-dimethyl sulfoxide, 10% SDS, 20% acetic acid) for 30 min at 37 °C. The amount of formazan was quantified spectrophotometrically at 570 nm. Cell viability was also assessed by nuclear morphology by staining with DAPI (Vectorshield, Burlingame, CA).

Western Blot Analysis—The cells in a 24-well plate were extracted from an SDS sample buffer (50 mM Tris, pH 7.5, 1% SDS, 2 mM EDTA, 1 mM Na₂VO₄, 50 mM NaF, 10 mM sodium β-glycerophosphate containing a complete protease inhibitor mixture (Roche, Applied Science)). The samples were resolved by 2–15% SDS-PAGE and transferred to nitrocellulose membranes. The filters were blocked with 5% nonfat dried milk in phosphate-buffered saline plus 0.5% Tween 20, followed by incubation with the primary antibodies of rabbit anti-peptide (CGPAAAGGGRTEA) AATYK antibody, anti-c-Jun, anti-phospho-c-Jun Ser-63/73 (Santa Cruz, CA), anti-phospho-Akt Ser-473, anti-p38, anti-phospho-p38 Thr180/Tyr182, anti-JNK, and anti-phospho-JNK Thr183/Tyr185 (Cell Signaling Technology, Beverly, MA). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Pierce) and detected using the ECL system (Amershams Biosciences).

Construction of Plasmids Encoding AATYK and Expression—The cDNAs encoding the FLAG-tagged C-terminal deletion mutant of AATYK were generated by PCR using full-length mouse AATYK cDNA in pCAGGS (18) as a template. Site-directed mutagenesis of AATYK was conducted by PCR using complementary sense and antisense oligonucleotides containing specific mutation. After digesting the parental DNA with Dpn 1, the PCR products were transformed into Escherichia coli. The clones were isolated, and the sequences were verified. The expression vectors were transfected by the calcium-phosphate method as previously described (18) with the following slight modification: the calcium-DNA precipitations were incubated with cells for 30 min at 2.5% CO₂.

Analysis of Apoptotic Cells Overexpressing AATYK—The expression vectors for FLAG-tagged AATYK and green fluorescent protein (ratio, 3:1) were co-transfected into CGCs as described previously (18). One day after transfection, apoptosis was induced by switching the medium to LK. Seventeen hours later, the cells were stained by the anti-FLAG and DAPI. Transfected cells expressing GFP and/or AATYK were assessed for apoptosis by nuclear morphology (pyknotic nuclei) as described previously (18).

RESULTS

Phosphorylation of AATYK Protein after Switching from High to Low KCl—To gain insight into the AATYK signaling cascade, we focused on the molecular changes in AATYK protein during LK-induced CGC apoptosis. As shown in Fig. 1A, the mobility shift of the AATYK protein was detected in HK after 7 days in culture with high KCl (25 mM) in a neurobasal medium supplemented with B27, CGCs were replaced for 2 hi in either an HK or LK medium alone or with EGTA (5 mM) (C); BAPTA (10 μM), thapsigargin (thap; 1 μM), nifedipine (nif; 5 μM), or α-conotoxin (α-con; 3 μM) (D); A23187 (200 μM), nimodipine (nim; 0.5 μM), BayK8644 (BayK; 1 μM) (E). Lysates extracted from the SDS sample buffer were subjected to Western blot analysis with anti-AATYK antibody.

FIGURE 1. AATYK is hyperphosphorylated during LK-induced CGC apoptosis and hypophosphorylated under survival HK conditions by L-type VDCCs-mediated Ca²⁺ influx. A, after 7 days in culture with high KCl (25 mM) in a neurobasal medium supplemented with B27, CGCs were replaced for 2 hi in either an HK or LK medium alone or with EGTA (5 mM) (C; BAPTA (10 μM), thapsigargin (thap; 1 μM), nifedipine (nif; 5 μM), or α-conotoxin (α-con; 3 μM) (D); A23187 (200 μM), nimodipine (nim; 0.5 μM), BayK8644 (BayK; 1 μM) (E). Lysates extracted from the SDS sample buffer were subjected to Western blot analysis with anti-AATYK antibody.

L-type Voltage-dependent Ca²⁺ Channels Mediate the Hypophosphorylation of AATYK in CGCs Cultured in HK—To elucidate the determinant(s) of the phosphorylation state of AATYK in HK and LK, we examined the effects of various pharmacological reagents on the molecular shift of AATYK on SDS-PAGE. As shown in Fig. 1C, treatment with extracellular Ca²⁺ chelator EGTA (5 mM) in HK did shift the AATYK band up to the LK-induced position, although there was no change in the banding position in LK. These data suggest that the intracellular Ca²⁺ level under HK culture conditions regulates the hypophosphorylation state of AATYK.

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To determine whether the hypophosphorylated state of AATYK in HK is mediated via Ca\(^{2+}\) signaling, we analyzed the inhibitory effects of reagents that influence the intracellular Ca\(^{2+}\) dynamics in CGCs (Fig. 1D). Treatment with BAPTA-AM (intracellular Ca\(^{2+}\) chelator), thapsigargin (inhibitor of ER Ca\(^{2+}\) ATPase), and α-conotoxin (antagonist of N-type VDCCs) did not affect the AATYK banding pattern in HK and LK. In contrast, nifedipine (antagonist of L-type VDCCs) induced the molecular shift in HK as did EGTA, but showed no effect in LK. Neither A23187 (Ca\(^{2+}\) ionophore) nor BayK8644 (agonist of L-type VDCCs) affected LK-induced phosphorylation (Fig. 1E). These results suggest that Ca\(^{2+}\) influx through L-type VDCCs is involved in the HK-induced hypophosphorylation of AATYK but not LK-induced hyperphosphorylation.

**AATYK Hyperphosphorylation in HK Is Mediated by Ca\(^{2+}\)-dependent Protein Phosphatase Activity**—To further characterize the Ca\(^{2+}\) signal-mediated hypophosphorylated state of AATYK in HK, we examined the effects of reagents that influence Ca\(^{2+}\)-dependent protein phosphorylation in CGCs. Treatment of CGCs with KN62 (inhibitor of Ca\(^{2+}\)-calmodulin-dependent protein kinase) in HK caused the AATYK band to shift up to the position induced by nimboline (L-type VDCC inhibitor) in HK or to the position induced in LK on SDS-PAGE (Fig. 2A), whereas it had no effect on the mobility of AATYK in LK. Similarly, treatment with cyclosporin A (inhibitor of protein phosphatase 2B/calcineurin) changed the hypophosphorylated position primarily in HK to the hyperphosphorylated position similar to that in LK. Cyclosporin A did not have any effect on the LK-induced molecular shift. These results suggest that the Ca\(^{2+}\)-dependent signaling pathway mediated via Ca\(^{2+}\)-calmodulin-dependent protein kinase and protein phosphatase 2B/calcineurin is involved in HK-induced AATYK hypophosphorylation but not in LK-induced AATYK hyperphosphorylation.

**AATYK Hyperphosphorylation in LK Is Inhibited by Treatment with the Glutamate Receptor Antagonist Kainate and the PKC-δ Inhibitor Rottlerin**—Because VDCC-mediated Ca\(^{2+}\) influx affected the HK-induced hypophosphorylation but not the LK-induced hyperphosphorylation of AATYK, we analyzed the contribution of glutamate receptors, another pathway for the Ca\(^{2+}\) influx in CGCs. Treatment with kainate (KA) in LK suppressed the switching of AATYK from hypophosphorylation state to hyperphosphorylation state in a dose-dependent manner (Fig. 2B). Although KA blocked the LK-induced molecular shift, it did not affect the binding pattern in HK. An additional AATYK band was detected between the band positions in HK and LK depending on the dose of KA (e.g. at 100 μM KA in Fig. 2B), indicating that AATYK in LK is hyperphosphorylated in at least two more sites than AATYK in HK. Neither AMPA nor NMDA showed a change in this molecular shift (Fig. 2C). Moreover, simultaneous treatment with DNQX or NBQX (blockers of AMPA/KA receptors), but not APV (blocker of NMDA receptors), released the suppression of LK-induced hyperphosphorylation by KA (Fig. 2D). These data indicate that the KA receptor activity possibly regulates the AATYK phosphorylation states.

We next examined the effects of other kinase inhibitors (Fig. 2E). Rottlerin (specific inhibitor of Ca\(^{2+}\)-independent and diacylglycerol-dependent protein kinase C-δ isoform, PKC-δ) blocked the LK-induced AATYK molecular shift (Fig. 2E), suggesting that PKC-δ is involved in the signaling mechanism underlying the LK-induced AATYK hyperphosphorylation. The conventional PKC inhibitor, Go6976, on the other hand, did not affect it (data not shown). Staurosporine (a broad spectrum kinase inhibitor) showed a slight inhibitory effect at 100 nM. Genistein (a tyrosine kinase inhibitor) induced no change.

None of the treatments of CGCs with p38 kinase inhibitor (10 μM; SB203580), Cyclin-dependent protein kinase 5 inhibitor (10 μM; roscovitine), mitogen-activated protein kinase kinase inhibitor (20 μM; PD98059), or phosphatidylinositol 3-kinase inhibitor (10 μM; LY294002) affected the molecular position of AATYK in either HK or LK (data not shown).

**Lithium Inhibits LK-induced AATYK Phosphorylation**—We next analyzed the effects of apoptosis-related reagents on the differential phosphorylation states of AATYK. The widely used mood-stabilizing drugs lithium and valproic acid (VAP) have been shown to confer neuroprotection against apoptotic stimuli (23–25). Treatment with lithium in LK blocked the LK-induced mobility shift of AATYK in a dose-dependent fashion (Fig. 3A). On the other hand, VAP had no significant effect on AATYK phosphorylation (Fig. 3B). None of the treatments of CGCs with caspase inhibitor (20 μM; Z-VAD), antioxidant (5 mM; N-acetyl L-cysteine), or IGF-1 (100 ng/ml) affected the molecular position of AATYK in either HK or LK (data not shown).

**AATYK Phosphorylation Is Concurrent with c-Jun Phosphorylation**—Phosphorylation of the transcription factor c-Jun, an immediate-early gene, is closely associated with CGC apoptosis (9). We analyzed the possible correlation between c-Jun and AATYK in terms of apoptosis-associated phosphorylation (Fig. 4).
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FIGURE 4. Correlation of AATYK and c-Jun phosphorylation. CGCs grown in NB plus B27 with HK for 7 days were replaced for 2 h in either an HK or LK medium alone or with treatment with nimodipine (nimo; 0.5 μM), KN62 (10 μM), kainic acid (KA; 100 μM), rottlerin (rot; 10 μM), or LiCl (Li; 20 mM), as indicated. Lysates extracted from the SDS sample buffer were subjected to Western blot analysis with anti-phospho Ser-63 and Ser-73-c-Jun antibody or anti-c-Jun antibody.

LK induced an increase in the amount of phosphorylated c-Jun, as did AATYK. Treatment with nimodipine and KN62 increased the phosphorylation of c-Jun, whereas treatment with kainate, rottlerin, and lithium in HK inhibited it. Cyclosporin A in HK also induced the phosphorylation of both c-Jun and AATYK (data not shown). These results suggest that the phosphorylation of AATYK coincides with that of c-Jun during the LK-induced apoptosis of CGCs.

AATYK Phosphorylation Is Associated with CGC Apoptosis—To study the functional relevance of AATYK phosphorylation and CGC apoptosis, we assayed cell viability by DAPI staining and under culture conditions that influence the phosphorylation states of AATYK (Fig. 5). CGCs undergoing apoptosis in LK conditions display characteristic chromatin condensation, allowing identification by DAPI staining as shown in Fig. 5A. The number of CGCs showing this apoptotic nuclear morphology increased in the presence of nimodipine in HK. In the presence of nimodipine in HK, AATYK reverted to the hyperphosphorylated state primarily in LK as shown in Figs. 1 and 2. On the other hand, lithium treatment in HK reduced the number of apoptotic CGCs. The same conditions (LK plus lithium) reversed the phosphorylation states to hyper- to hypophosphorylated as shown in Fig. 3. These results suggest that the phosphorylation states of AATYK are closely associated with CGC apoptosis.

Next, we quantified cell viability by MTT assay at 24 h after treatment with the agents that affected AATYK phosphorylation (Fig. 5B). Fifty to 60% of CGCs became apoptotic in the HK medium, as previously reported (22). Treatment with nimodipine, KN62, and Cyclosporin A reduced the survival rate of CGCs in HK in a dose-dependent manner, whereas these treatments did not have an effect in LK at the same doses. On the other hand, kainate, rottlerin, and lithium protected CGCs from LK-induced apoptosis in a dose-dependent manner. These results suggest that AATYK phosphorylation states correlate with HK-induced survival and the LK-induced apoptosis of CGCs.

AATYK Is Phosphorylated at Multiple Sites—AATYK was hyperphosphorylated at multiple sites under HK culture conditions. We examined whether the self-kinase activity of AATYK is involved in LK-induced hyperphosphorylation. FLAG-tagged full-length recombinant AATYK proteins (AATYK-wt) (Fig. 6A) exogenously expressed in CGCs were phosphorylated under HK conditions (Fig. 6B). However, kinase-deficient AATYK (AATYK-mc), which has a point mutation at the catalytic site (Asp-206 to Val, D206V), did not show an LK-induced molecular shift (Fig. 6B), suggesting that the kinase activity of AATYK itself is essential for LK-induced phosphorylation. To determine the LK-induced phosphorylation sites, we generated a series of C-terminal truncation mutants with (wt) and without (mc) kinase activity (Fig. 6A). When the full-length AATYK (1,317 amino acid residues) was expressed in 293T cells, the molecular size of wild-type AATYK-wt was larger than that of the kinase-deficient AATYK-mc (Fig. 6C). Treatment with phospha tea led to the same mobility in both constructs (data not shown); the difference in the mobility observed is therefore dependent on self-kinase activity. By utilizing this property, we screened the C-terminal truncation constructs (up to the 1184, 883, 537, 465, and 360 residues) for phosphorylation candidate sites (Fig. 6, B and C). As a result, the molecular shift disappeared by deleting up to the 465th residue. Interestingly, the kinase-active wt series of more than 625 residues showed multiple banding patterns in comparison with the kinase-deficient mutant counterparts (mc): i.e. single in AATYK-537-wt versus mc, double in AATYK-625-wt versus -mc, and more than double in AATYK-883-wt versus -mc. These results indicate that AATYK has at least two phosphorylation sites, one located between residues 465 and 537 and the other between residues 537 and 625. Although AATYK-883-wt seemed to have more than two bands, the kinase-deficient AATYK-883-mc also showed two bands. Taken together, these results suggest that two candidate sites within residues 465–625 are phosphorylated by AATYK kinase activity, whereas other potential sites are phosphorylated by the intrinsic kinases of 293T cells other than AATYK.

There are four phosphorylation candidate sites (Ser-480, Tyr-487, Ser-558, and Ser-566) among the 471–600 residues (Fig. 7A) of AATYK (predicted by software, Prosite and NetPhos). We generated seven substitution mutants of AATYK-625 (S480G, Ser-480 to Gly; S480A, Ser-480 to Ala; S480D, Ser-480 to Asp; Y487F, Tyr-487 to Phe; Y487D, Tyr-487 to Asp; S558A, Ser-558 to Ala; S556A, Ser-566 to Ala) and analyzed the molecular shift of these constructs expressed in the 293T cells (Fig. 7). The control, AATYK-625-wt, expressed in the 293T cells showed three bands; the upper band (third band) was major, the middle band (second band) was minor, and the lower band (first band) was barely detectable (Fig. 7B). In the mutants S480G and S480A, the second band became major instead of the third band. The mutant Y487F showed a similar banding pattern to that of S480G and S480A. These results suggest that the efficiency of phosphorylation at one of the two phosphorylation sites seems to decrease in these mutants. In contrast, the mutants S480D and Y487D primarily showed the third band, suggesting that all phosphorylation sites in these mutants are fully phosphorylated. This mutant effect may be because negatively charged Asp residue mimics a phosphate moiety at Ser-480 or Tyr-487 in a phosphorylated state at these sites. In the mutants S558A and S566A, the first band, which was located at the same position of the kinase-deficient construct, became detectable, although the other two bands were more abundantly observed.

We next generated the double mutant S558A/S566A and triple mutant S480G/S558A/S566A and analyzed their banding patterns in the 293T cells. The double mutant S558A/S566A had a banding pattern where the first band was major but the other bands were faintly observed, whereas the triple mutant S480G/S558A/S566A showed almost a single band pattern at the first position.

To analyze the effects of these phosphorylation mutations on switching the phosphorylation states in CGCs, we introduced these single, double, and triple substitution mutations into the FLAG-tagged full-length AATYK (AATYK-wt, AATYK-mc, AATYK-S480G, AATYK-S558A/S566A, AATYK-S480G/S558A/S566A, AATYK-S480D, and AATYK-Y487D) (Fig. 8A). As a result, AATYK-wt showed LK-induced molecular shift in CGCs, but the kinase-deficient mutant AATYK-mc did not, as expected (Fig. 8B). In contrast, the triple-Ser mutant AATYK-S480G/S558A/S566A was defective in LK-induced molecular shift, although it had the active kinase domain (wt). On the other hand, the AATYK-S480D and Y487D mutants displayed an LK-induced molecular shift, but of a reduced nature. It is notable that the AATYK-S480G/S558A/S566A mutant induced a slight shift by nimodipine.
treatment in HK (Fig. 8C). These results suggest that in response to apoptotic LK and/or the blockade of L type-VDCC, AATYK is hyperphosphorylated in a combination of these three Ser residues in CGCs, although additional residue(s) are likely phosphorylated in the presence of nimodipine (Fig. 8C). This phosphorylation state change appears to require at least the self-kinase activity.

**DISCUSSION**

In this study, we investigated the phosphorylation states of AATYK in regulating the HK-induced survival and LK-induced apoptosis of CGCs. In addition, we identified the amino acid residues that are phosphorylated in LK and that regulate the apoptotic activity of AATYK.

HK seems to provide the conditions necessary for membrane depolarization of neurons, thereby leading to an increase in the probability of
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The phosphatidylinositol 3-kinase-Akt pathway is important for CGC survival mediated by neurotrophic factors such as IGF-1 (15). IGF-1 rescues CGCs from apoptosis induced by LK (6). However, in our LK culture, IGF treatment did not block the hyperphosphorylation of AATYK (data not shown). There is the possibility that IGF receptors are activated by the insulin-containing B27 supplement used in our serum-free medium (27) so that additional IGF-1 has no more effects on AATYK phosphorylation. Under this condition, LK-induced CGC apoptosis was induced and accompanied by the hyperphosphorylation of AATYK. Furthermore, the phosphatidylinositol 3-kinase inhibitor LY294002 did not induce the hyperphosphorylation of AATYK in HK (data not shown). Therefore, AATYK hypophosphorylation in HK is likely independent of the phosphatidylinositol 3-kinase-Akt pathway in the present experimental condition.

LK probably causes non-depolarization of the plasma membrane. This state of activity deprivation may lead to cell death. It would be interesting to determine whether there is any synergistic or complementary signaling between the underlying pathways of the hypophosphorylation of AATYK in response to survival HK and apoptotic LK, respectively. One such candidate is Ca$^{2+}$ signaling, because HK-induced hypophosphorylation is Ca$^{2+}$-calmodulin-dependent. However, LK-induced hyperphosphorylation was unaffected by pharmacological reagents that alter Ca$^{2+}$ dynamics, such as EGTA, BAPTA-AM, A23187, L-type VDCC agonist and antagonists, N-type VDCC inhibitor, and thapsigargin. Our data indicate that kainate, but not NMDA or AMPA, blocks the LK-induced phosphorylation of AATYK. In addition, kainate protected CGCs from LK-induced apoptosis, although more than 100 μM kainate had a cytotoxic effect on CGC survival in HK (data not shown). These results suggest that the kainate receptor-mediated pathway gives rise to HK-like depolarizing, survival conditions, thereby leading to the hypophosphorylation of AATYK. Taken together, hypophosphorylation is controlled by the Ca$^{2+}$ signaling pathway, whereas hyperphosphorylation is regulated by a pathway in which Ca$^{2+}$ signaling is not the major determinant. Increasing evidence for lithium as a neuroprotective agent has been reported (24, 25, 28), and in our experiments, it suppressed the LK-induced hyperphosphorylation of AATYK (Fig. 3). Inhibition of inositol monophosphatase
PKC-related pathway.

Among the kinase inhibitors we examined, rottlerin, an inhibitor of PKC-δ, blocked the LK-induced AATYK hyperphosphorylation. PKC-δ has been implicated in apoptosis (36–38), is highly expressed in the brain (39), and is induced after ischemic insults (40–42). A specific PKC inhibitor, bisindolylmaleimide, blocks cyclin D1 expression and the apoptotic activation. A

In conclusion, we have demonstrated that AATYK switches its phosphorylation states in response to survival HK and apoptotic LK conditions. The hypophosphorylation state in HK is likely to be regulated by the lithium-sensitive, PKC-δ-related pathway. Switching to the opposite state could be conducted by pharmacological agents (nimodipine, kainate, lithium, etc.) depending on the KCl concentration in the culture medium, by which CGC survival could be improved. Moreover, LK-induced CGC apoptosis could be reduced by overexpressing the phosphorylation site deletion mutant (S480G/S558A/S666A) as well as the kinase-deficient mutant of AATYK. Therefore, our study indicates that the switching two phosphorylation states of AATYK is closely related to CGC survival and apoptosis.

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