Journal of Neurochemistry
Lippincott Williams & Wilkins, Inc., Philadelphia

Kainic Acid-Induced Neurotrophic Activities in Developing Cortical Neurons

*Yi-Hsuan Lee, †Kwang-Ming Fang, ‡Chuen-Mao Yang, §Hwa-Min Hwang, ‡Chi-Tso Chiu, and ‖Wuhong Tsai

**Department of Physiology and †Graduate Institute of Medicine, Taipei Medical College, Taipei; Departments of ‡Pharmacology and §Anatomy, Chang Gung University, Taoyuan, Taiwan, Republic of China; and ‖Antiviral Research Lab, CDER, Food and Drug Administration, Rockville, Maryland, U.S.A.*

Abstract: Using primary cultured cortical neurons from embryonic rat brains, we elucidated an α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainic acid (KA) receptor-mediated neuroprotective mechanism through actions of nerve growth factor (NGF) in developing neurons. Neurotoxicity of KA in early days in vitro neurons was quite low compared with the mature neurons. However, pretreatment with anti-NGF antibody or TrkA inhibitor AG-879 profoundly raised KA toxicity. Furthermore, KA stimulation resulted in an increase of Trk A expression and phosphorylation, which was blocked not only by the AMPA/KA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione and AG-879, but also by the phospholipase C inhibitor U73122 and the intracellular calcium chelator BAPTA. A study of polyphosphoinositide turnover showed that KA-stimulated phospholipase C (PLC) activity was directly triggered by the AMPA/KA receptor activity, but not by the activity of TrkA or other excitatory amino acid receptor subtypes. Sources of KA-increased intracellular calcium levels were contributed by both extracellular calcium influx and intracellular calcium release and were partially sensitive to guanosine 5′-*O*-(2-thiodiphosphate). These results indicate that in developing cortical neurons, activation of AMPA/KA receptors by KA may induce expression, followed by activation of TrkA via PLC signaling and intracellular calcium elevation and hence increase reception of NGF on KA-challenged neurons. A G protein-coupled AMPA/KA receptor may be involved in these metabotropic events for neuronal protection. **Key Words:** Kainic acid—α-Amino-3-hydroxy-5-methyl-4-isoxazole propionate/kainic acid receptors—Neuroprotection—Phospholipase C—Nerve growth factor—Calcium.
J. Neurochem. **74,** 2401–2411 (2000).

In the mammalian brain, excitatory amino acids (EAAs), mainly glutamate and aspartate, function as neurotransmitters mediating the vast majority of excitatory synaptic transmissions and also as neurotrophic factors for neuronal development (for review, see McDonald, 1993). In neonatal brains, the ionotropic EAA receptors NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainic acid (KA) receptors display a sequential participation in neuronal excitation (Ben-Ari et al., 1997). When reaching the adult stage, these ionotropic receptors mainly mediate rapid synaptic transmissions, and an excessive degree of receptor activation may lead to neuronal cell death, known as glutamate excitotoxicity (Olney, 1986). KA in particular is a potent neurotoxin commonly used to induce severe seizures and a pattern of hippocampal damage similar to Ammon's horn sclerosis in adult rats (Nadler et al., 1978; Lothman and Collins, 1981). In developing brains, however, administration of KA failed to produce neurotoxicity in various experimental systems (Campochiaro and Coyle, 1978; Albala et al., 1984; Holmes and Thompson, 1988; Sperber et al., 1991). Instead, low doses of EAAs appear to exert trophic effects such as increases in survival and neurite outgrowth of cultured neurons derived from hippocampus, cerebellum, and spinal cord (Balázs and Hack, 1990; Cohen-Cory et al., 1991). A critical level of EAA receptor activation could be required for normal development: Underactivation can retard or disrupt normal development, whereas over-

Received September 8, 1999; revised manuscript received January 12, 2000; accepted February 7, 2000.

Address correspondence and reprint requests to Dr. Y.-H. Lee at Department of Physiology, Taipei Medical College, 250 Wu-Hsing Street, Taipei, Taiwan, Republic of China. E-mail: hsuan@tmc.edu.tw

Abbreviations used: AG-879, 3,5-di-*tert*-butyl-4-hydroxybenzylidenecyanthioacetamide; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; L-AP4, 2-amino-4-phosphono-*S*-butanoic acid; (±)-AP5, (±)-2-amino-5-phosphonovaleric acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DIV, days in vitro; EAA, excitatory amino acid; EBSS, Earle's balanced saline solution; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); genistein, 4′,5,7-trihydroxyisoflavone; IPn, inositol phosphates; KA, kainic acid; LDH, lactate dehydrogenase; MCPG, (±)-α-methoxy-(4-carboxyphenyl)glycine; NGF, nerve growth factor; NT, neurotrophin; PI, phosphatidylinositol; PLC, phospholipase C; poly-PI, polyphosphoinositide; TTX, tetrodotoxin; U73122, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione.

excitation can lead to neuronal injury and destruction (McDonald and Johnston, 1993).

The fact that AMPA/KA receptors are more permeable to Ca^{2+} in developing neurons implies that neurotrophic activity of the receptor could be mediated by calcium entry (Hume et al., 1991; Monyer et al., 1991; Kohler et al., 1993; Pellegrini-Giampietro et al., 1997). In 1994, we demonstrated that calcium-dependent, 2-amino-4-phosphono-*S*-butanoic acid (L-AP4)-insensitive polyphosphoinositide (poly-PI) turnover, resulting from activation of phospholipase C (PLC) to generate second messenger inositol triphosphate, was elicited by AMPA and KA specifically in premature cultured neurons (Lee et al., 1994). This information suggests that the developmental role of the AMPA/KA receptor is based on its regulation of calcium and inositol trisphosphate, the major signals regulating electrical excitability and neurite outgrowth via intercellular diffusion (Kater and Mills, 1991; Kandler and Katz, 1995).

Neuronal development requires a group of proteins known as neurotrophic factors in the extracellular environment to regulate survival, growth, morphological plasticity, or synthesis of proteins for differentiated functioning of neurons (Hefti et al., 1993). The neurotrophins (NTs) are a family of related neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor, NT-3, and NT-4/5. NGF, which is responsible for synaptic targeting and nerve regeneration, binds to its target cells bearing its receptors, namely, TrkA with high affinity and $p75^{NTR}$ with low affinity, to autophosphorylate their tyrosine residues (Bothwell, 1991; Barbacid, 1995). Several investigations have shown that interruption of NGF-mediated signaling in neonatal animals could result in irreversible degeneration or mistargeting of developing neurons. Conversely, exogenous NGF treatment was found to prevent the naturally occurring cell death of developing sympathetic neurons (Johnson et al., 1980; Fagan et al., 1996).

Interactions between EAAs and NT-mediated cell signaling in developing brains are complex and largely remain unclear. AMPA/KA receptors have drawn particular attention because their activation has been associated with increased expressions of NT mRNAs (Zafra et al., 1990; Cohen-Cory et al., 1991). Therefore, this article presents our studies in elucidating the signaling mechanism of KA-induced neurotrophic activity in developing rat cortical neurons. Sequential events occurring on KA stimulation, such as intracellular calcium elevation, TrkA expression and phosphorylation, involvement of G protein, poly-PI turnover, and neuronal survival, were investigated.

MATERIALS AND METHODS

Materials

Pregnant female Sprague–Dawley rats were obtained from the National Institute of Experimental Animal Research (Taipei, Taiwan, Republic of China). Basal medium Eagle and fetal calf serum were obtained from Life Technology (Gaithersburg, MD, U.S.A.). KA, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), (±)-2-amino-5-phosphonovaleric acid [(±)-AP5], 4′,5,7-trihydroxyisoflavone (genistein), 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]-hexyl]-1*H*-pyrrole-2,5-dione (U73122), and tetrodotoxin (TTX) were obtained from Research Biochemicals International (Natick, MA, U.S.A.). 3,5-Di-*tert*-butyl-4-hydroxybenzylidenecyanthioacetamide (AG-879) was obtained from Biomol (Plymouth Meeting, PA, U.S.A.). The Vectastain ABC kit and the horseradish peroxidase-chemiluminescence western blotting kit were obtained from Vector Laboratories (Burlingame, CA, U.S.A.) and Pierce (Rockford, IL, U.S.A.), respectively. *myo*-[^{3}H]Inositol and Hybond ECL nitrocellulose membranes were obtained from Amersham Life Science (Little Chalfont, Bucks, U.K.). Protein A-Sepharose for immunoprecipitation was from Pharmacia Biotech (Uppsala, Sweden). Polyclonal antibodies to NGF and TrkA and monoclonal antibodies to phosphotyrosine were purchased from Chemicon (Temecula, CA, U.S.A.), Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), and Upstate Biotechnology (Lake Placid, NY, U.S.A.), respectively. Most reagents applied in the following experiments were dissolved in Earle's balanced saline solution (EBSS; 117 m*M* NaCl, 1 m*M* NaH_2PO_4, 5.3 m*M* KCl, 26 m*M* $NaHCO_3$, 0.8 m*M* $MgSO_4$, 1.8 m*M* $CaCl_2$, and 5.6 m*M* D-glucose). The stock solution of AG-879 was prepared in dimethyl sulfoxide at 1 m*M*. All common reagents were purchased from Sigma (St. Louis, MO, U.S.A.) or Merck (Darmstadt, Germany).

Primary culture of cortical neurons

Primary cortical cultures were prepared as described previously (Lee et al., 1994) with minor modifications. In brief, cerebral cortex was dissected from fetal rats at embryonic day 16–18. Cortical tissue was mechanically dissociated through a 14-gauge metal needle, washed three times, and finally plated onto poly-L-lysine-coated culture plates at a density of $\sim 1 \times 10^6$ cells per 35-mm-diameter culture dish or 5×10^5 cells per well of 24-well culture plates with basal medium Eagle supplemented with sodium bicarbonate (13.1 m*M*), D-glucose (13.9 m*M*), L-glutamine (2.0 m*M*), and heat-inactivated fetal calf serum (20%). Cells were initially incubated for 30–45 min in a humidified incubator with 5% CO_2 at 37°C for monolayer attachment, after which the medium was replaced by serum-free basal medium Eagle. Cells were thereafter maintained in 5% CO_2 at 37°C. Cultures obtained under these conditions appeared to contain mostly neuronal cells [>85% (Lee et al., 1994)].

Quantitative assay of neuronal cell death

Neuronal cell death was visualized qualitatively with microscopic observations and then quantified by measuring the activity of lactate dehydrogenase (LDH) released from damaged cells into the extracellular fluid as described previously (Deupree et al., 1996). Cultured neurons were equilibrated with newly changed basal medium Eagle for 1 h, followed by enzyme inhibitors or antagonist pretreatment for 20 min and KA stimulation for 5–15 min or 30–45 min as the brief or prolonged treatment, respectively. The culture medium of the treated neurons was harvested 12–16 h after the stimulants were removed. For the LDH assay, 0.5 ml of medium was incubated with 0.2 mg of β-NADH in 2.4 ml of 0.1 *M* phosphate buffer for 5–15 min at room temperature. The absorbance at a wavelength of 340 nm was measured for 3 min immediately after 0.1 *M* sodium pyruvate was added to the mixture.

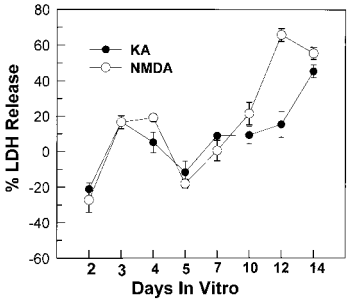

FIG. 1. EAA neurotoxicity in developing cortical neurons. Cultured cortical neurons at various DIVs were stimulated by 500 μM KA (●) or NMDA (○) for 5–15 min, and the degree of cell death was quantified by LDH release analysis. Data are expressed as percent increase of LDH release over the basal level of culture in each DIV and are mean ± SEM (bars) values (n = 4).

The unit activity of LDH was defined as the decrease of A_{340} in one min, multiplied by 1,000, in 1 ml of sample.

Western blot analysis of TrkA

Cultured neurons with designated treatment were harvested with ice-cold homogenizing buffer (50 m*M* Tris-HCl containing 1 m*M* EDTA, 1 m*M* sodium orthovanadate, trypsin inhibitor, 0.5 m*M* phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin, pH 7.4). Cells were sonicated and centrifuged at 100,000 *g* for 30 min to obtain the crude membrane fraction in the pellet. To determine factors that affect TrkA expression, 20 μg of the pellet protein was separated onto 7% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a Hybond ECL nitrocellulose membrane, probed with the anti-TrkA antibody and biotin-labeled anti-mouse IgG, and visualized by horseradish peroxidase-reactive chemiluminescence. To quantify phosphorylated TrkA further, 30 μg of membrane protein was lysed in lysis buffer [homogenizing buffer plus 0.5% Igepal CA-630 (Sigma)] and incubated with 20 μl of washed protein A and 5 μg of anti-phosphotyrosine for 12 h at 4°C. After incubation, the immune complexes were washed three times with lysis buffer and twice with homogenizing buffer. Tyrosine-phosphorylated proteins were then eluted from Sepharose beads by adding 30 μl of 2× Laemmli's sample buffer, boiled for 5 min, and collected in the supernatant after centrifugation. TrkA was then detected in the eluted proteins by western blot analysis using anti-TrkA antibody. The relative density of the protein band in the western blot was further analyzed using the Digital Science Electrophoresis Documentation and Analysis System (Eastman Kodak Co., Rochester, NY, U.S.A.).

Poly-PI turnover analysis

Poly-PI turnover in cultured neurons was determined by prelabeling cells with 1 μCi of *myo*-[^{3}H]inositol/10^6 cells for 6 h in 5% CO_2 at 37°C, followed by three washes for 10 min each with EBSS containing 10 m*M* LiCl and agonist stimulation for 45 min with a final volume of 0.6 ml. All incubations described above were in 5% CO_2 at 37°C. The stimulation was terminated by 4 volumes of chloroform/methanol (2:1 vol/vol) to extract phosphatidylinositol (PI) and inositol phosphates (IPn). The aqueous phase of the mixture, which contained labeled IPn, was eluted from the AG1-X8 (Bio-Rad) anion exchange column by 400 m*M* sodium formate in 100 m*M* formic acid. The organic phase contains labeled PI, which was air-dried and directly counted for radioactivity with a scintillation counter. Levels of poly-PI turnover were expressed as the [^{3}H]IPn/[^{3}H]PI ratio, which normalizes variations in incorporation of [^{3}H]inositol in cultures.

Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured in cortical neurons cultured on coverslips with the calcium-sensitive dye fura-2/AM as described by Yang et al. (1994). Cells were incubated with 1 ml of basal medium Eagle's containing 5 μM fura-2/AM for 45 min at 37°C. At the end of the loading period, the coverslip was washed twice with physiological buffer solution containing 125 m*M* NaCl, 5 m*M* KCl, 1.8 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 0.5 m*M* NaH_2PO_4, 5 m*M* $NaHCO_3$, 10 m*M* HEPES, and 10 m*M* glucose, pH 7.4. Cells were incubated in physiological buffer solution for another 30 min to complete dye deesterification. The coverslip was inserted at a 45° angle to the excitation beam into a quartz cuvette and placed in the thermostatted holder of an SLM 8000C spectrofluorometer. Fluorescence of Ca^{2+}-bound and unbound fura-2 was measured by rapidly alternating the dual excitation wavelengths between 340 and 380 nm and electronically separating the resultant fluorescence signals at an emission wavelength of 510 nm. The values of the ratio (R) of the fluorescence at the two wavelengths were computed and used to calculate changes in $[Ca^{2+}]_i$. Ratios of maximal (R_{max}) and minimal (R_{min}) fluorescence of fura-2 were determined by addition of ionomycin (10 m*M*) in the presence of physiolog-

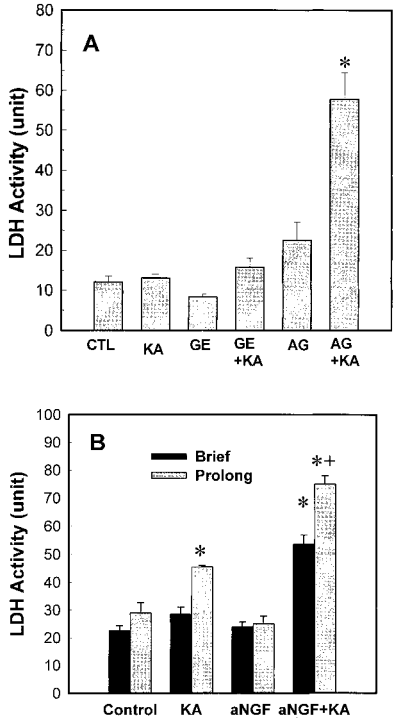

FIG. 2. Effect of TrkA inhibitors and anti-NGF on KA toxicity in developing cortical neurons. **A:** Cultured cortical neurons at 4 DIV were pretreated with 10 μM genistein (GE) or 10 μM AG-879 (AG) for 30 min, followed by 500 μM KA stimulation for 15 min or an equal volume of EBSS as the control (CTL). **B:** Neurons at 4 DIV were pretreated with anti-NGF antibody (aNGF; 4 μg/ml), followed by brief (15-min) or prolonged (30-min) 500 μM KA stimulation. $^*p < 0.05$, compared with their respective CTL; $^+p < 0.05$, compared with the respective KA stimulation group.

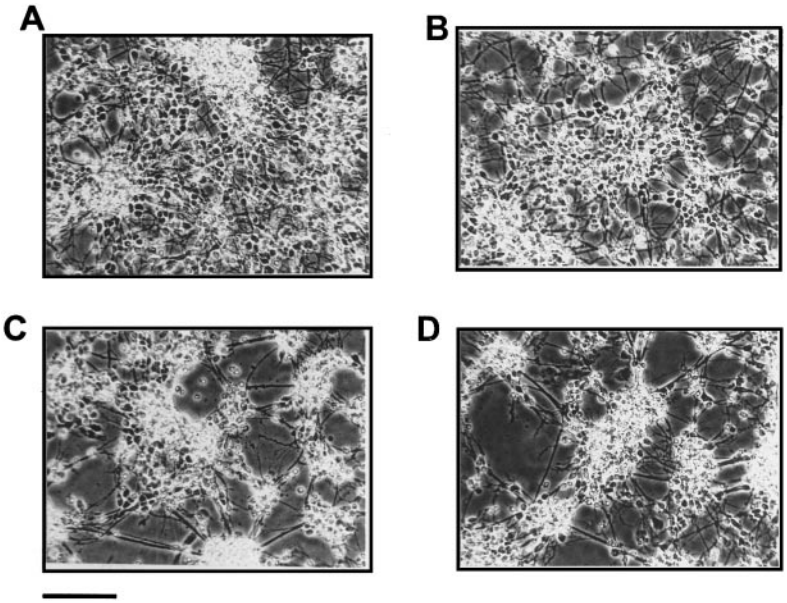

FIG. 3. Phase-contrast photomicrographs of cortical neurons 16 h after 15-min exposure to (**A**) EBSS or (**B**) 500 μM KA alone or pretreated with (**C**) anti-NGF antibody (4 μg/ml) or (**D**) AG-879 (10 μM). Bar = 50 μm.

ical buffer solution containing 5 mM Ca^{2+} and by adding 5 mM EGTA at pH 8 in Ca^{2+}-free physiological buffer solution, respectively. The $[Ca^{2+}]_i$ was obtained from the following equation: $[Ca^{2+}]_i = K_D \times \beta \times (R - R_{min})/(R_{max} - R)$, in which $K_D = 224$ nM and $\beta = 6.9352$ were assumed.

RESULTS

Neurotoxicity of KA in cortical neurons during development

At first, to know how cultured neurons at various developing stages would respond to KA, neurons at selected days in vitro (DIVs) were tested for their susceptibilities to 500 μM KA stimulation. In parallel, stimulation with NMDA, known as the most excitotoxic EAA, was also conducted for comparison. Using LDH release assay to quantify cell death, it was found that neurotoxicity of both EAA agonists was low in the 2–10 DIV period and increased thereafter (Fig. 1). During the 2–10 DIV period, the toxicity of both KA and NMDA was relatively low. When cultured neurons reached maturation, significant neurotoxicity appeared slightly earlier in the NMDA group (at 12 DIV) than in the KA group (at 14 DIV). This result indicates that EAAs at high concentration are indeed not as excitotoxic to developing neurons as to well-developed neurons. One of the reasons for this phenomenon could be due to a neuroprotective mechanism triggered by EAA stimulation to survive EAA-challenged neurons. The following studies were hence conducted based on this hypothesis.

Effects of tyrosine kinase inhibitors and anti-NGF on neuroprotective effect of KA in developing neurons

Neurotrophic factors, such as NGF, are known to be neuroprotective via activation of the TrkA receptor. We therefore examined its involvement in AMPA/KA receptor functions by pretreating 4–6 DIV cultured neurons with anti-NGF antibody or TrkA inhibitors. KA-stimulated neurons were first pretreated with 10 μM genistein, a general tyrosine kinase inhibitor, at a maximal concentration to be nontoxic to neurons but yet effective at inhibiting tyrosine kinase activity. LDH release of 4 DIV neurons was slightly more than with KA stimulation alone (Fig. 2A). Second, pretreatment with 10 μM AG-879, a specific inhibitor for TrkA autophosphorylation with an IC_{50} of 10 μM (Levitzki and Gazit, 1995), gave a profound increase of KA toxicity. The same effect was observed when extracellular NGF was neutralized by anti-NGF antibody during both brief (15-min) and prolonged (30-min) KA stimulation, in which KA neurotoxicity was markedly revealed (Fig. 2B). This microscopic observation also showed that 4 DIV cortical neurons subjected to KA stimulation alone survived and were as healthy as the vehicle-treated neurons 16 h after treatment (Fig. 3A and B). However, KA-stimulated cortical neurons pretreated with AG-879 and anti-NGF showed severe neuronal loss and shrinkage of neurites (Fig. 3C and D). These results indicate that the activation of AMPA/KA receptors by KA, while exerting its neurotoxic effect, may also induce a NGF-mediated TrkA activation to protect developing cortical neurons.

Involvement of the NMDA receptor in KA-induced neuroprotective effect

The KA stimulation may result in an increase of extracellular glutamate content to activate further other glutamate receptor subtypes such as the NMDA receptor, which could be the source of the neuroprotective activity of KA. To examine this possibility, the NMDA receptor antagonist (±)-AP5 was applied onto the cultured neuron before the KA stimulation with both brief and prolonged duration. Figure 4 shows that (±)-AP5 strongly

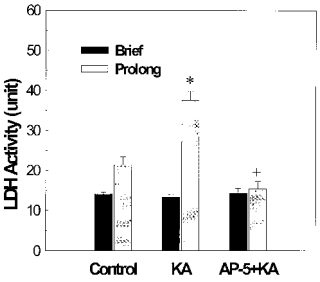

FIG. 4. Effect of NMDA receptor antagonist on KA neurotoxicity. Neurons at 4 DIV were pretreated with (±)-AP5 (500 μM) for 10 min, followed by brief (15-min) or prolonged (30-min) 500 μM KA stimulation. $^{*}p < 0.05$, compared with their respective controls. $^{+}p < 0.05$, compared with the respective KA stimulation group.

affected prolonged KA stimulation-induced cell death but not brief KA stimulation-maintained cell survival. Judging from this result, it is conceivable that the NMDA receptor activity during KA stimulation mainly contributes to excitotoxicity but not to the neuronal protection.

KA-induced TrkA expression and phosphorylation is calcium- and PLC-dependent

Because the signaling pathway of NGF is initiated by tyrosine phosphorylation of TrkA and/or $p75^{NTR}$, we first examined whether the KA stimulation can induce expression and activation of TrkA in developing cortical neurons. At first, KA stimulation was found to stimulate both TrkA expression and TrkA phosphorylation in the first 5-min period (Fig. 5A). However, the degree of TrkA phosphorylation seems to be much greater than the TrkA up-regulation as the stimulation duration approaches 30 min. Figure 5B quantified the intensity of the detected TrkA and phosphorylated TrkA in Fig. 5A, indicating that KA stimulation initially increased TrkA expression and equivalent TrkA phosphorylation and subsequently induced massive TrkA phosphorylation. Because the 30-min KA stimulation exerted a maximal effect on TrkA phosphorylation, we then used this stimulation duration to examine the mechanism involved.

Regarding the induction of TrkA expression, Fig. 5C shows that the KA-induced TrkA expression was sensitive to the PLC inhibitor U73122 and the intracellular cal-

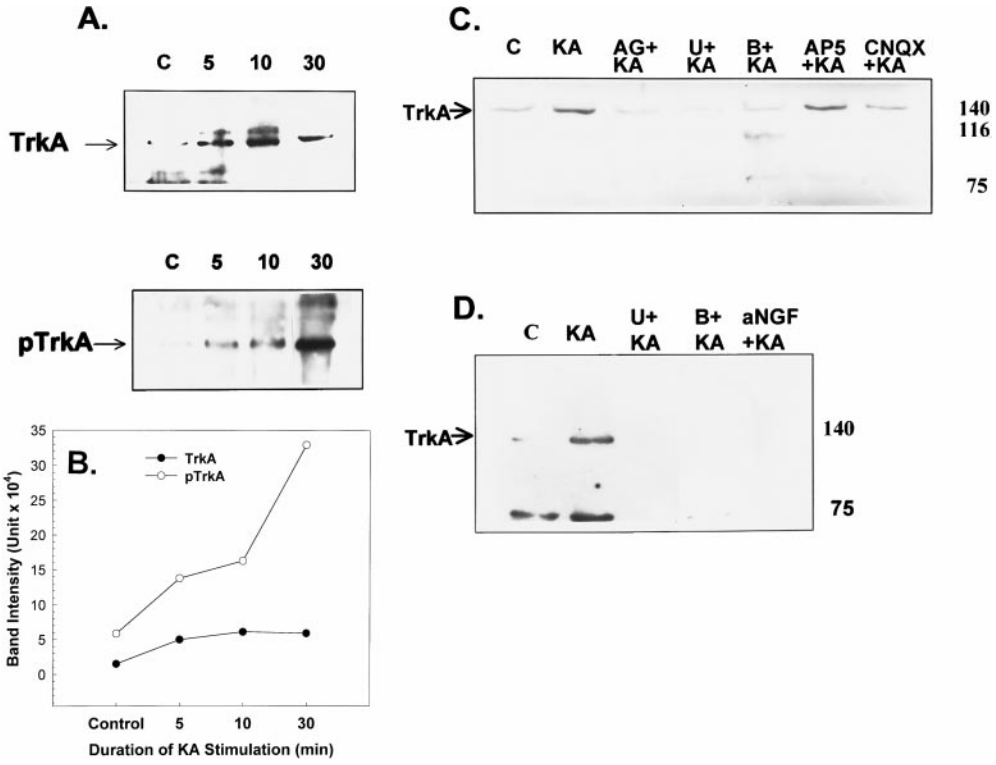

FIG. 5. KA-induced TrkA expression and phosphorylation in developing cortical neurons. **A:** TrkA and phospho-TrkA (pTrkA), which was immunoprecipitated with the anti-phosphotyrosine antibody and western-blotted with the anti-TrkA antibody, were detected in neurons after 5, 10, and 30 min of 500 μM KA stimulation. C, EBSS-treated group. The position of TrkA (140 kDa) is indicated. **B:** Densitometric analysis. **C:** 4 DIV neurons were pretreated with EBSS (C), BAPTA (B; 4 mM), AG-879 (AG; 10 μM), or U73122 (U; 10 μM) for 30 min or (±)-AP5 (500 μM) or CNQX (500 μM) for 10 min, followed by 500 μM KA stimulation for 30 min. Membrane protein of neurons was western-blotted with the anti-TrkA antibody as indicated by an arrow. **D:** Cultured cortical neurons were pretreated with B (4 mM), anti-NGF antibody (aNGF; 4 mg/ml), or U (10 μM) for 30 min, followed by 500 μM KA stimulation for 30 min. Membrane protein of neurons was immunoprecipitated with the anti-phosphotyrosine antibody and western-blotted with the anti-TrkA antibody as indicated by an arrow.

cium chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA). KA failed to induce TrkA expression when neurons were pretreated with AG-879, suggesting that TrkA expression may depend on its own activation. KA-induced TrkA expression was completely abolished by the AMPA/KA receptor antagonist CNQX but not affected by the NMDA receptor antagonist (±)-AP5. These results ensure that the neurotrophic effect of KA is specifically via AMPA/KA receptors and not via the NMDA receptor. On the other hand, tyrosine phosphorylation of TrkA was examined by immunoprecipitating crude membrane protein of cultured neurons with anti-phosphotyrosine antibody and western-blotted with the anti-TrkA antibody. Figure 5D shows that KA profoundly increased tyrosine phosphorylation of TrkA and that BAPTA, the PLC inhibitor U73122, and the anti-NGF antibody reduced this effect. These results suggest that the induction of TrkA phosphorylation by KA is via increase of intracellular calcium, possibly contributed by the PLC activity.

KA-induced poly-PI turnover in developing neurons

As the PLC activity is involved in the KA-induced TrkA expression and activation in developing neurons, we further examined the level of poly-PI turnover on KA stimulation. Indeed, Fig. 6A shows that 500 μM KA increased poly-PI turnover approximately onefold above the control level. This effect was only blocked by the AMPA/KA receptor antagonist CNQX but not by the NMDA receptor antagonist (±)-AP5 or by the metabotropic glutamate receptor antagonist (±)-α-methyl-(4-carboxyphenyl)glycine (MCPG). A voltage-gated sodium channel blocker, TTX, generally used to block quantal release of neurotransmitters, also had no inhibition of the KA-induced PI response at a concentration of 10 μM, which is effective for blocking glutamate or dopamine release in the culture system used here (data not shown). These results suggest that KA-induced poly-PI turnover is mediated directly by the activation of AMPA/KA receptors, but not by other EAA receptors or other neurotransmitter systems. Furthermore, the KA-induced poly-PI response was inhibited by 10 μM U73122 and 4 m*M* BAPTA, reflecting the involvement of calcium-dependent PLC activation (Fig. 6B). Lastly, application of NGF onto 4 DIV neurons without KA stimulation showed no significant induction of poly-PI turnover (Fig. 6C), suggesting that this effect does not come from activation of the NGF receptor. Anti-NGF antibody and AG-879 also had no effect on KA-induced poly-PI turnover, supporting the hypothesis that the NGF receptor activation is the result, but not the cause, of KA-triggered PLC activation.

KA-induced $[Ca^{2+}]_i$ increase in developing neurons

Because KA-mediated neurotrophic activities in the above studies were found to be calcium-dependent, we also investigated factors contributing to the KA-mediated $[Ca^{2+}]_i$ increase using fluorescence spectrophotometry. In 4 DIV cortical neurons, application of 500 μM KA markedly raised $[Ca^{2+}]_i$ in 45 s. The KA-increased $[Ca^{2+}]_i$, while being completely blocked by CNQX, was only slightly reduced (~30%) by (±)-AP5 (Fig. 7A and B). This result indicates that KA increased $[Ca^{2+}]_i$ mainly via primary activation of AMPA/KA receptors and not by a secondary activation of the NMDA receptor. In fact, a lack of synaptic maturation in early developing neurons makes it impossible for KA to activate the NMDA receptor by stimulating glutamate release. In addition, pathways involved in the net elevation of KA-increased $[Ca^{2+}]_i$ also include the voltage-gated calcium channel (blocked by 10 μM nifedipine), intracellular calcium release (reduced by 10 μM dantrolene), and PLC activity (partially reduced by 20 and 50 μM U73122) (Fig. 7C and D). The potencies of these inhibitors in

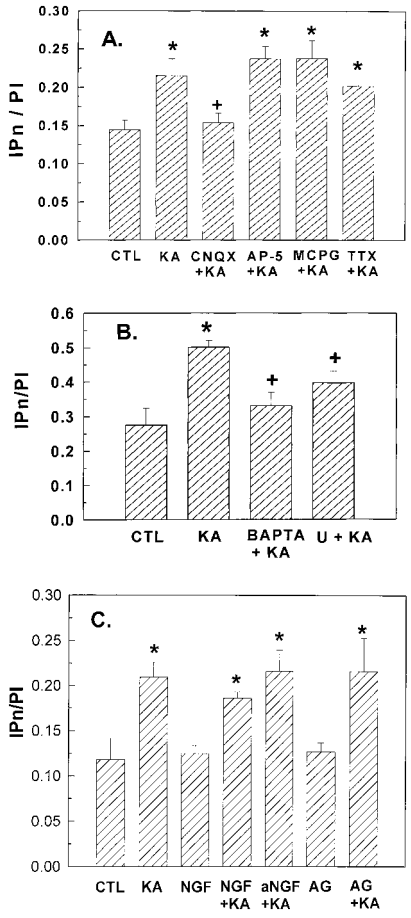

FIG. 6. KA-induced poly-PI turnover in developing cortical neurons. Cultured cortical neurons at 4 DIV were allowed to incorporate *myo*-[^{3}H]inositol for 6 h, followed by a 20-min pretreatment with the following reagents before a 45-min stimulation with KA (500 μM). CTL, control. **A:** EAA antagonists CNQX, (±)-AP5, and MCPG (500 μM). A sodium channel blocker, TTX (1 μM), was also tested. **B:** Intracellular calcium chelator BAPTA (4 m*M*) and PLC inhibitor U73122 (U; 10 μM). **C:** NGF (100 ng/ml), anti-NGF antibody (aNGF; 4 μg/ml), and TrkA inhibitor AG-879 (AG; 10 μM). IPn/PI represents the level of poly-PI turnover as described in Materials and Methods. Data are mean ± SEM (bars) values (n = 4). $^*p < 0.05$, compared with CTL; $^+p < 0.05$, compared with the KA-stimulated group.

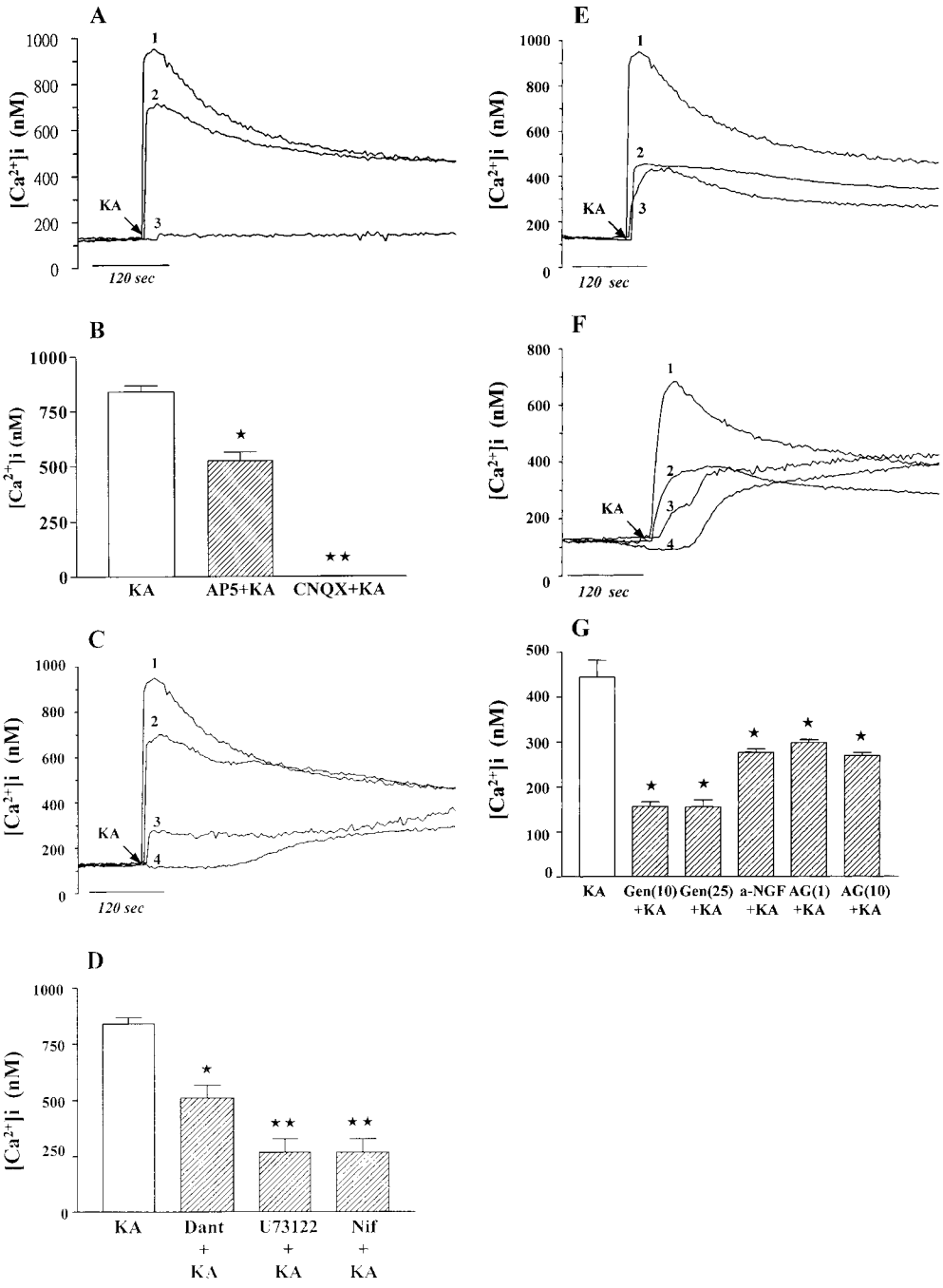

FIG. 7. Sources of KA-stimulated $[Ca^{2+}]_i$ increase. The cortical neurons on glass coverslips were loaded with 5 μM fura-2, and fluorescent detection of intracellular calcium was carried out for 600 s in each experiment in a dual excitation wavelength spectrophotometer. **A** and **B:** 4 DIV neurons were preincubated with the AMPA/KA receptor antagonist CNQX (500 μM) or the NMDA receptor antagonist (±)-AP5 (500 μM) at 37°C for 10 min, and KA (final concentration 500 μM) was added at the time as indicated (arrow) and incubated with cells for 600 s. In A, trace 1, KA stimulation only; trace 2, (±)-AP5 + KA; trace 3, CNQX + KA. **C** and **D:** Neurons were preincubated with the voltage-sensitive calcium channel blocker nifedipine (Nif), the intracellular calcium release blocker dantrolene (Dant), or the PLC inhibitor U73122 at 37°C for 30 min, and KA was added at the time as indicated (arrow) and incubated with cells for 600 s. In C, trace 1, KA stimulation alone; trace 2, Dant + KA; trace 3, U73122 + KA; trace 4, Nif + KA. **E–G:** Neurons were preincubated with genistein (Gen) at 10 or 25 μM, AG-879 (AG) at 1 or 10 μM, or anti-NGF antibody (a-NGF; 4 μg/ml) at 37°C for 30 min, and KA was added at the time as indicated (arrow) and incubated with cells for 600 s. In E, trace 1, KA stimulation alone; trace 2, Gen (10 μM) + KA; trace 3, Gen (25 μM) + KA. In F, trace 1, KA stimulation alone; trace 2, a-NGF + KA; trace 3, AG (1 μM) + KA; trace 4, AG (10 μM) + KA. A, C, E, and F showed representative profiles, and B, D, and G showed mean ± SEM (bars) values (n = 5) of the peak values of $[Ca^{2+}]_i$ changes for each experimental conditions. $^*p < 0.05$, $^{**}p < 0.005$, compared with the KA group.

inhibiting KA-induced $[Ca^{2+}]_i$ are in the order nifedipine = U73122 > dantrolene, implying that KA-induced voltage-gated calcium channel opening could be a prerequisite for triggering PLC activity and intracellular calcium release. Furthermore, applications of genistein, AG-879, and anti-NGF significantly reduced KA-increased $[Ca^{2+}]_i$ in the neuronal population (Fig. 7E–G) suggesting that KA-induced tyrosine phosphorylation and TrkA activation also account in part for the KA-increased $[Ca^{2+}]_i$.

Involvement of G protein in KA-induced metabotropic activities in developing neurons

KA-induced neurotrophic activities seems to involve a metabotropic activity directly mediated by AMPA/KA receptors. Therefore, we further examined whether the KA-induced metabotropic activity is coupled to G proteins. Guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS), a nonhydrolyzable GDP analogue used to inactivate G proteins even in intact cells (Sim et al., 1995; Centemeri et al., 1997), was applied to the poly-PI turnover and $[Ca^{2+}]_i$ studies. Figure 8 shows that the KA-stimulated $[Ca^{2+}]_i$ increase and poly-PI turnover increase were both reduced significantly by 1 m*M* GDPβS, a concentration that had no effect on the calcium ionophore A23187-increased poly-PI turnover (data not shown). These results strongly suggest that a G protein-coupled AMPA/KA receptor could be present in developing neurons to mediate neurotrophic functions.

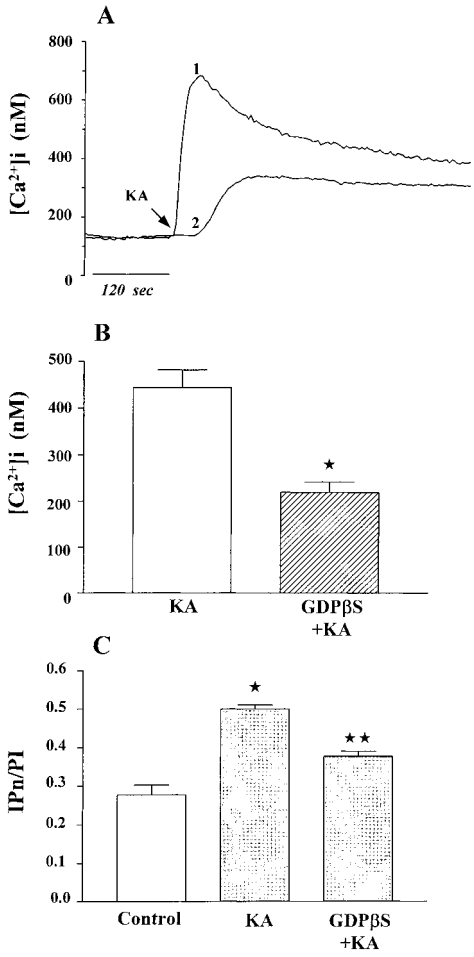

FIG. 8. Effect of GDPβS on KA-increased $[Ca^{2+}]_i$ and poly-PI turnover in developing cortical neurons. Cortical neurons at 4 DIV were preincubated with 1 m*M* GDPβS, followed by 500 μM KA stimulation for either 600 s for the $[Ca^{2+}]_i$ study (**A** and **B**) or 45 min for the poly-PI turnover study (**C**). *$p < 0.05$, compared with the control group; **$p < 0.05$, compared with the KA group.

DISCUSSION

In this article, many observations suggest that KA appears to be neuroprotective to developing cortical neurons by stimulating NGF-mediated neurotrophic activities, including anti-NGF-sensitive neuronal survival and increased expression and phosphorylation of the TrkA receptor. KA-induced PLC activity and $[Ca^{2+}]_i$ increase were found to be the intracellular signals necessary for triggering neurotrophic events.

Specificity of KA stimulation

KA at 500 μM used in this study could cause not only activation of the AMPA/KA receptor, but also stimulation of nonvesicular release of glutamate (Szatkowski et al., 1990). The latter event may further activate the NMDA receptor. However, the activity of the NMDA receptor was found not to contribute to the neurotrophic effect of KA because of the following observations: (a) Figure 4 shows that (±)-AP5 cannot reduce neuronal survival and, in fact, protect neurons under prolonged KA stimulation. (b) Figure 5C shows that (±)-AP5 cannot reduce KA-induced TrkA expression. (c) Figure 6A shows that (±)-AP5 cannot reduce KA-induced poly-PI turnover. Therefore, the NMDA receptor activated on KA stimulation is neurotoxic but not neurotrophic.

AMPA/KA receptors versus NGF–TrkA activity

A high concentration of EAAs, especially KA, is believed to be neurotoxic owing to its overactivation of EAA receptors and inhibition of EAA transporter (Hoehn and White, 1990), which leads to massive depolarization of CNS neurons. However, our study demonstrated that KA at 500 μM was in fact neuroprotective and able to induce significant levels of TrkA expression and phosphorylation. This finding coincides with the study showing that KA can increase the NGF mRNA level in developing neurons (Zafra et al., 1990). TrkA is known as one of the early responsive gene products, which can be rapidly and transiently induced by environmental factors. In this study, TrkA expression was also found to be increased rapidly in the first 5 min of KA stimulation, which provides more receptors for NGF to bind. Therefore, AMPA/KA receptors may play a crucial role during neuronal development, in which cell viability should be precisely controlled for differentiation and maturation. This finding also explains why the neurotrophic activity of EAA receptors is more prominent in the CNS at the developing stage or the injury repair stage

because neurons under these two circumstances show a high requirement for neurotrophic activities for survival. In developing cortical neurons, neurotrophic and signaling events elicited by KA were also proved to be specifically mediated by AMPA/KA receptors but not by cross-reacting with the NMDA receptor. This finding is important for differentiating the role of each EAA receptor subtype in neuronal development. Furthermore, as the constitutive release of NGF from cultured neurons is known to be highly important for maintaining their own viability, it is conceivable that AMPA/KA receptors may mediate NGF release in developing neurons for both basal survival and protections from neurotoxic insults.

PLC versus TrkA receptor in AMPA/KA receptor-mediated neurotrophic functions

The intracellular signaling involved in AMPA/KA receptor-mediated neurotrophic function appears to be triggered by PLC. In fact, some NTs were found to increase poly-PI turnover via their PLC-coupled receptors. It has been reported that brain-derived neurotrophic factor and NT-3, but not NGF, activate TrkB and TrkC receptors, respectively, to induce poly-PI turnover (Widmer et al., 1993; Marsh and Palfrey, 1996). Cultured cortical neurons used in this study were also found to give no PI response when treated with NGF at a neuroprotective concentration. The tyrosine kinase inhibitor genistein and a TrkA inhibitor had no effect on the KA-induced PI response, providing confirmation that the PLC signaling pathway is upstream, not downstream, of the NGF–TrkA activity. It should be noted that excessive activation of PLC by prolonged KA stimulation still led to cell death of 4 DIV neurons (authors' unpublished data). Therefore, the PLC activity may in fact regulate neuronal survival by a two-stage action: an initial stage for triggering the NGF–TrkA signaling to protect neurons and a late stage for a massive increase of $[Ca^{2+}]_i$ to kill neurons.

Role of calcium in AMPA/KA receptor-mediated neurotrophic function

In this study, roles of intracellular calcium in KA-mediated neurotrophic functions were elucidated, including initiation of PLC activity, activation of TrkA possibly by stimulating NGF release, and its participation in the neurotrophic function of TrkA. That multiple sources of $[Ca^{2+}]_i$ can be elicited by KA also indicates that AMPA/KA receptors may play a crucial role in manipulating calcium homeostasis in developing CNS. It is interesting to know that activation of TrkA seems to increase $[Ca^{2+}]_i$ further in neurons. TrkA has been demonstrated to increase calcium uptake and intracellular calcium accumulation in other cell types such as human 3T3 cells and C6-2B glioma cells (De Bernardi et al., 1996; Jiang et al., 1997). Although how the increase of $[Ca^{2+}]_i$ by TrkA protects neurons remains unclear, it has been suggested that depolarization may also increase neuronal survival by making neurons more responsive to neurotrophic factors (Meyer-Franke et al., 1995) or by enhancing de novo synthesis of these factors (Ghosh et al., 1994). In addition, calcium-dependent, paxilline-sensitive potassium channels (BK channels) have been found to be key effectors for the NT-regulated neuronal activity (Holm et al., 1997). However, it should be noted that genistein, while inhibiting the KA-increased $[Ca^{2+}]_i$, had little effect on cell death, suggesting that the survival event could be specifically mediated by TrkA. This phenomenon implies that at least part of the genistein-reduced $[Ca^{2+}]_i$ is not essential for the neurotrophic activity of the AMPA/KA receptor.

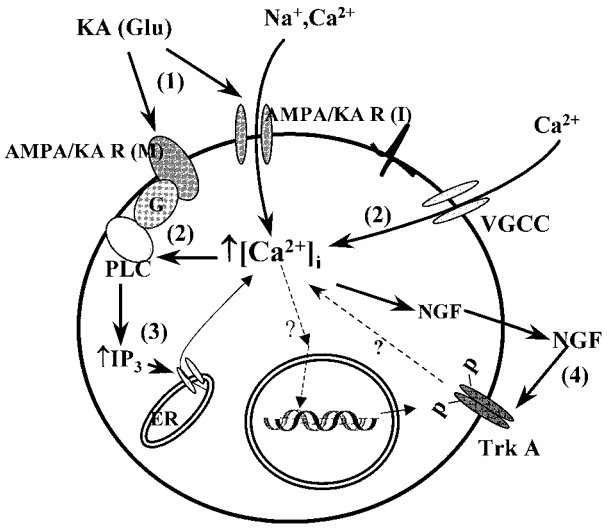

FIG. 9. Signaling mechanism of AMPA/KA receptor (R)-mediated neurotrophic activities in developing cortical neurons. Step 1: KA activates both ionotropic (I) and metabotropic (M) AMPA/KA Rs in developing cortical neurons. Step 2: Activation of I receptor opens the voltage-gated calcium channel (VGCC) and leads to calcium influx to activate calcium-dependent PLC, whereas activation of M receptor activates G protein-linked PLC. Step 3: Activated PLC catalyzes poly-PI turnover and in turn increases inositol trisphosphate (IP_3)-gated calcium release from endoplasmic reticulum (ER) into cytoplasm. Step 4: Elevated $[Ca^{2+}]_i$ induces both TrkA expression and NGF release to up-regulate the NGF–TrkA signaling, which in turn protects KA-challenged neurons.

Metabotropic activities of AMPA/KA receptors in developing cortical neurons

One of the most striking findings in this study is the effect of GDPβS on KA-induced $[Ca^{2+}]_i$ elevation and poly-PI turnover. This information suggests that KA may act, at least in part, through a G protein-coupled metabotropic receptor to exert its neurotrophic functions. Calcium-dependent metabotropic activities induced by KA or AMPA were found not only in developing cortical neurons, but also in other developing neural cells such as oligodendrocyte progenitor cells (Lee et al., 1994; Liu et al., 1997). An immunolocalization study has also indicated that the AMPA receptor may exist at the nonsynaptic region to regulate neuronal maturation in developing cerebellar cortex (Ripellino et al., 1998). Moreover, Rodriguez-Moreno and Lerma (1998) reported data for the presence of a G protein-coupled presynaptic KA receptor in

hippocampal neurons, which is linked to the PLC signaling pathway like the metabotropic AMPA/KA receptor we found here in developing cortical neurons. Although the metabotropic AMPA/KA receptor presented here may function differently from the presynaptic KA receptor, which was found to down-regulate the inhibitory postsynaptic current via inhibition of GABA release, it is conceivable that these two receptors may be the same receptor with differential functions in different developmental stages. Molecular cloning and structural determination are hence needed to prove the existence of the metabotropic AMPA/KA receptor to support this hypothesis.

Conclusions

On the basis of results obtained from this study, a molecular mechanism regarding the signal transduction pathway of AMPA/KA receptor-mediated neurotrophic activity is proposed as shown in Fig. 9. When developing neurons are stimulated by KA or endogenous glutamate, both ionotropic and metabotropic AMPA/KA receptors are activated (step 1). Activation of the ionotropic receptor opens voltage-gated calcium channels to allow more calcium flux in to activate calcium-dependent PLC, whereas the metabotropic receptor activates G protein-linked PLC (step 2). Activation of PLC leads to an increase of poly-PI turnover, in which inositol trisphosphate is generated and acts on intracellular calcium stores to release more calcium into the cytoplasm (step 3). Both TrkA expression and autocrine/paracrine actions of NGF are induced in a calcium-dependent manner in KA-challenged neurons, which result in TrkA activation to protect neurons from KA neurotoxicity (step 4). In conclusion, AMPA/KA receptors, with their ionotropic and metabotropic activities, may play a protective role in the survival of cortical neurons through neural development.

Acknowledgment: This work is supported by grant NSC 87-2314-B-038-038 from the National Science Council, Taiwan. We thank Dr. Jang-Yen Wu and Dr. Gean-Chuan Yeh for critical review of the manuscript. The expert photograph preparation by Pao-Yao Chiu is greatly appreciated.

REFERENCES

Albala B. J., Moche S. L., and Okada R. (1984) Kainic acid-induced seizures: a developmental study. *Dev. Brain Res.* **13,** 139–148.

Balázs R. and Hack N. (1990) Trophic effects of excitatory amino acids in the developing nervous system. *Adv. Exp. Med. Biol.* **268,** 221–228.

Barbacid M. (1995) Neurotrophic factors and their receptors. *Curr. Opin. Cell Biol.* **7,** 148–155.

Ben-Ari Y., Khazipov R., Leinekugel X., Caillard O., and Caiarsa J. L. (1997) $GABA_A$, NMDA and AMPA receptors: a developmentally regulated 'ménage à trois.' *Trends Neurosci.* **20,** 523–529.

Bothwell M. (1991) Keeping track of neurotrophin receptors. *Cell* **65,** 915–918.

Campochiaro P. and Coyle J. T. (1978) Ontogenetic development of kainate neurotoxicity: correlates with glutamatergic innervation. *Proc. Natl. Acad. Sci. USA* **75,** 2025–2029.

Centemeri C., Bolego C., Abbracchio M. P., Cattabeni F., Puglisi L., Burnstock G., and Nicosia S. (1997) Characterization of the Ca^{2+} responses evoked by ATP and other nucleotides in mammalian brain astrocytes. *Br. J. Pharmacol.* **121,** 1700–1706.

Cohen-Cory S., Dreyfus C. F., and Black I. B. (1991) NGF and excitatory neurotransmitters regulate survival and morphogenesis of cultured cerebellar Purkinje cells. *J. Neurosci.* **11,** 462–471.

De Bernardi M. A., Rabins S. J., Colangelo A. M., Brooker G., and Mocchetti I. (1996) TrkA mediates the nerve growth factor-induced intracellular calcium accumulation. *J. Biol. Chem.* **271,** 6092–6098.

Deupree D. L., Tang X. W., Yarom M., Dickman E., Kirch R. D., Schloss J. V., and Wu J. Y. (1996) Studies of NMDA- and non-NMDA-mediated neurotoxicity in cultured neurons. *Neurochem. Int.* **29,** 255–261.

Fagan A. M., Zhang H., Landis S., Smeyne R. J., Silos-Santiago I., and Barbacid M. (1996) TrkA, but not trkC receptors are essential for survival of sympathetic neurons in vivo. *J. Neurosci.* **16,** 6208–6218.

Ghosh A., Carnahan J., and Greenberg M. E. (1994) Requirement for BDNF in activity-dependent survival of cortical neurons. *Science* **263,** 1618–1623.

Hefti F., Knusel B., and Lapchak P. A. (1993) Protective effects of nerve growth factor and brain-derived neurotrophic factor on basal forebrain cholinergic neurons in adult rats with partial fimbrial transections. *Prog. Brain Res.* **98,** 257–263.

Hoehn K. and White T. D. (1990) Glutamate-evoked release of endogenous adenosine from rat cortical synaptosomes is mediated by glutamate uptake and not by receptors. *J. Neurochem.* **54,** 1716–1724.

Holm N. R., Christophersen P., Olesen S. P., and Gammeltoft S. (1997) Activation of calcium-dependent potassium channels in rat brain neurons by neurotrophin-3 and nerve growth factor. *Proc. Natl. Acad. Sci. USA* **94,** 1002–1006.

Holmes G. L. and Thompson J. L. (1988) Effect of kainic acid on seizure susceptibility in the developing brain. *Dev. Brain Res.* **39,** 51–59.

Hume R. I., Dingledine R., and Heinemann S. (1991) Identification of a site in glutamate receptor subunits that controls calcium permeability. *Science* **253,** 1028–1031.

Jiang H., Ulme D. S., Dickens G., Chabuk A., Lavarreda M., Lazarovici P., and Guroff G. (1997) Both p140 (trk) and p75 (NGFR) nerve growth factor receptors mediate nerve growth factor-stimulated calcium uptake. *J. Biol. Chem.* **272,** 6835–6837.

Johnson E. M., Gorin P. D. Jr., Brandeis L. D., and Pearson J. (1980) Dorsal root ganglion neurons are destroyed by exposure in utero to maternal antibody to nerve growth factor. *Science* **210,** 916–918.

Kandler K. and Katz L. C. (1998) Coordination of neuronal activity in developing visual cortex by gap junction-mediated biochemical communication. *J. Neurosci.* **18,** 1419–1427.

Kater S. B. and Mills L. R. (1991) Regulation of growth cone behavior by calcium. *J. Neurosci.* **11,** 891–899.

Kohler M., Burnashev N., Sakmann B., and Seeburg P. H. (1993) Determinants of Ca^{2+} permeability in both TM1 and TM2 of high affinity kainate receptor channels: diversity by RNA editing. *Neuron* **10,** 491–500.

Lee Y.-H., Deupree D. L., Chen S.-C., Kao L.-S., and Wu J.-Y. (1994) Role of Ca^{2+} in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-mediated polyphosphoinositide turnover in primary neuronal cultures. *J. Neurochem.* **62,** 2325–2332.

Levitzki A. and Gazit A. (1995) Tyrosine kinase inhibition: an approach to drug development. *Science* **267,** 1782–1788.

Liu H. N., Molina-Holgado E., and Almazan G. (1997) Glutamate-stimulated production of inositol phosphates is mediated by Ca^{2+} influx in oligodendrocyte progenitors. *Eur. J. Pharmacol.* **338,** 277–287.

Lothman E. W. and Collins R. C. (1981) Kainic acid-induced limbic seizures. Metabolic behavioral, electroencephalographic and neuropathological correlates. *Brain Res.* **218,** 299–318.

Marsh H. N. and Palfrey H. C. (1996) Neurotrophin-3 and brain-derived neurotrophic factor activate multiple signal transduction events but are not survival factors for hippocampal pyramidal neurons. *J. Neurochem.* **67,** 952–963.

McDonald J. W. and Johnston M. V. (1993) Excitatory amino acid neurotoxicity in the developing brain. *NIDA Res. Monogr.* **133,** 185–205.

Meyer-Franke A., Kaplan M. R., Pfrieger F. W., and Barres B. A. (1995) Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. *Neuron* **15,** 805–819.

Monyer H., Seeburg P. H., and Wisden W. (1991) Glutamate-operated channels: developmentally early and mature forms arised by alternative splicing. *Neuron* **6,** 799–810.

Nadler J. V., Perry B. W., and Cotman C. W. (1978) Intraventricular kainic acid preferentially destroys hippocampal pyramidal cells. *Nature* **271,** 676–677.

Olney J. W. (1986) Inciting excitotoxic cytocide among central neurons. *Adv. Exp. Med. Biol.* **203,** 631–645.

Pellegrini-Giampietro D. E., Gorter J. A., Bennett M. V. L., and Zukin R. S. (1997) The GluR2 (GluR-B) hypothesis: Ca^{2+}-permeable AMPA receptors in neurological disorders. *Trends Neurosci.* **20,** 464–470.

Ripellino J. A., Neve R. L., and Howe J. R. (1998) Expression and heteromeric interactions of non-*N*-methyl-D-aspartate glutamate receptor subunits in the developing and adult cerebellum. *Neuroscience* **82,** 485–497.

Rodriguez-Moreno A. and Lerma J. (1998) Kainate receptor modulation of GABA release involves a metabotropic function. *Neuron* **20,** 1211–1218.

Sim L. J., Selley D. E., and Childers S. R. (1995) In vitro autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5′-[γ-[^{35}S]thio]-triphosphate binding. *Proc. Natl. Acad. Sci. USA* **92,** 7242–7246.

Sperber E. F., Haas D. Z., Stanton P. K., and Moshe S. L. (1991) Resistance of the immature hippocampus to seizure-induced synaptic reorganization. *Dev. Brain Res.* **60,** 88–93.

Szatkowski M., Barbour B., and Attwell D. (1990) Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. *Nature* **348,** 443–446.

Widmer H. R., Kaplan D. R., Rabin S. J., Beck K. A., Hefti F., and Knüsel B. (1993) Rapid phosphorylation of phospholipase Cγ1 by brain-derived neurotrophic factor and neurotrophin-3 in cultures of embryonic rat cortical neurons. *J. Neurochem.* **60,** 2111–2123.

Yang C. M., Hsia H. C., Hsieh J. T., Ong R., and Luo S. F. (1994) Bradykinin-stimulated calcium mobilization in cultured canine tracheal smooth muscle cells. *Cell Calcium* **16,** 59–70.

Zafra F., Hengerer B., Leibrock J., Thoenen H., and Lindholm D. (1990) Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. *EMBO J.* **9,** 3545–3550.