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Cell cycle-dependent regulation of kainate-induced inward currents in microglia

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Abstract

Microglia are reported to have α-amino-hydroxy-5-methyl-isoxazole-4-propionate/kainate (KA) types. However, only small population of primary cultured rat microglia (approximately 20%) responded to KA. In the present study, we have attempted to elucidate the regulatory mechanism of responsiveness to KA in GMIR1 rat microglial cell line. When the GMIR1 cells were plated at a low density in the presence of granulocyte macrophage colony-stimulating factor, the proliferation rate increased and reached the peak after 2 days in culture and then gradually decreased because of density-dependent inhibition. At cell proliferation stage, approximately 80% of the GMIR1 cells exhibited glutamate (Glu)- and KA-induced inward currents at cell proliferation stage, whereas only 22.5% of the cells showed responsiveness to Glu and KA at cell quiescent stage. Furthermore, the mean amplitudes of inward currents induced by Glu and KA at cell proliferation stage (13.8 ± 3.0 and 8.4 ± 0.6 pA) were significantly larger than those obtained at cell quiescent stage (4.7 ± 0.8 and 6.2 ± 1.2 pA). In the GMIR1 cells, KA-induced inward currents were markedly inhibited by (*RS*)-3-(2-carboxybenzyl) willardiine (UBP296), a selective antagonist for KA receptors. The KA-responsive cells also responded to (*RS*)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl) propanoic acid (ATPA), a selective agonist for GluR5, in both GMIR1 cells and primary cultured rat microglia. Furthermore, mRNA levels of the KA receptor subunits, GluR5 and GluR6, at the cell proliferation stage were significantly higher than those at the cell quiescent stage. Furthermore, the immunoreactivity for GluR6/7 was found to increase in activated microglia in the post-ischemic hippocampus. These results strongly suggest that microglia have functional KA receptors mainly consisting of GluR5 and GluR6, and the expression levels of these subunits are closely regulated by the cell cycle mechanism.

Keywords: Microglia; Kainate; Granulocyte–macrophage colony-stimulating factor; Whole-cell patch clamp; Real-time quantitative RT-PCR

There is increasing evidence that glutamate (Glu) is one of the important molecules mediating the neuron-to-microglia communication in both physiological and pathological states [1–5]. We have previously reported that microglia express functional ionotropic Glu receptors, mainly α-amino-hydroxy-5-methyl-isoxazole-4-propionate (AMPA)/kainate (KA) types. Although the activation of these receptors enhanced the release of proinflammatory cytokines including tumor necrosis factor-α, only 20% of the primary cultured rat microglia could elicit inward currents after the application of KA [5]. However, the reason why the KA-responsive population of the microglia is so small. Gottlieb and Matute [6] have reported that the levels of Glu receptor subunits expressed in the microglia peaked between 3 and 7 days after transient forebrain ischemia, a time when strong microglial activation and proliferation were observed in the CA1 subfield of the hippocampus [7]. Furthermore, it is also known that a marked expression of cyclin D1, a key regulator of cell cycle progression, was followed by microglial proliferation [7,8]. This may suggest that the expression of Glu receptors in the microglia is closely linked with the cell cycle. To investigate this

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possibility, we examined the responses of the microglia to KA at different stages of cellular proliferation using the GMIR1 rat microglial cell line, which proliferates in response to granulocyte–macrophage colony-stimulating factor (GM-CSF) [9]. In the present study, we therefore provide evidence that a cell cycle-dependent regulation of both the response to KA and the expression level of KA receptor subunits exist in the microglia.

Materials and methods

Cell culture. GMIR1, an immortalized microglial clone, was established from a rat primary culture using a nonenzymatic and non-virus-transformed procedure [9,10]. The medium to maintain the GMIR1 cells was Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Wako Pure Chemical Industries, Ins., Osaka, Japan), 5 μg/ml insulin, 0.17% $NaHCO_3$, 0.2% glucose, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. 0.2 ng/ml rat recombinant GM-CSF (Genzyme, Cambridge, MA, USA) was supplemented in the culture medium to maintain the GMIR1 cells because these cells stop proliferating without it. The GMIR1 cells were grown in an atmosphere of 10% CO_2 at 37 °C and the medium was changed every 2–3 days. Every 2 months, new cells were started to avoid transformation. Primary cultured rat microglia were isolated from mixed glial cell cultures from the cerebral cortex of 3-day-old Wistar rats on the 14 day as reported previously [11]. Cultures were maintained in Eagle's MEM containing 10% FBS, 0.2% $NaHCO_3$, 2 mM glutamine, 0.2% glucose, 25 μg/ml insulin, 5000 U/ml penicillin, and 5 mg/ml streptomycin.

Electrophysiology. Whole-cell recordings were made as reported previously [2,5]. Microglial cells were whole-cell clamped using a patch pipette containing (in mM): CsCl 100, Na_2ATP 3, HEPES 5, $CaCl_2$ 1, $MgCl_2$ 4, EGTA 5, and *N*-methyl-D-glucamine (NMDG) 10. The pH of the solution was adjusted to 7.2 with NMDG. The pipette resistance was 5–8 MΩ. The external solution contained (in mM): KCl 2.5, NaCl 110, $CaCl_2$ 3, $BaCl_2$ 6, glucose 15, and HEPES 5, and the pH were adjusted to 7.4 with NMDG. Patch clamp recordings were obtained using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) in voltage clamp mode. Na^+ free solutions were substituted NaCl for NMDG. The external KA or drugs were applied rapidly using the Y tube technique, which allows for the complete exchange of the external solution surrounding a cell within 20 ms. pH of KA solution was adjusted to 7.4 with 1 N NaOH. The experiments were performed at room temperature. Many GMIR1 cells showed a small round shape under phase-contrast microscopy. We recorded small rounded cells for electrophysiological recordings. KA, Glu, and (*RS*)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl) propanoic acid (ATPA) were purchased from Sigma (St. Louis, MO, USA). (*RS*)-3-(2-carboxybenzyl) willardiine (UBP296) was purchased from Wako Pure Chemical Industries.

BrdU incorporation. The GMIR1 cells were plated 10^4 per one cover glass coated with polyethyllenimine and then stained with a BrdU immunohistochemistry kit (EMD Biosciences, Inc., La Jolla, CA, USA) according to the manufacturer's protocol. The images were obtained with a microscope (Akisionscope, Carl Zeiss) using a 40× water immersion objective. Images were transferred to a personal computer and analyzed using the Image J software program (v 3.04). A statistical analysis was carried out using ANOVA. A value of $P < 0.05$ was considered to indicate statistical significance.

Real-time quantitative RT-PCR. Total RNAs were prepared from 2 to 2.5×10^6 cells with a RNeasy RNA purification kit (Qiagen, Hilden, Germany). To avoid any contamination of genomic DNA, cytoplasmic RNAs were isolated and DNase was added according to the manufacturer's protocol. RT minus controls were run to confirm the presence of genomic contamination. First strand cDNA synthesized from 1 mg of total RNA with random hexamer primers and oligo (dT) primers was used as template in each reaction. Syber green based real-time RT-PCR was performed with DyNAmo SYBER Green 2-step qRT-PCR kit (Finnzymes, Espoo, Finland). Rotor Gene 3000 (Corbett Research, Mortlake, Australia) was used for the signal detection. PCR was performed using 1× master mix, 0.5 μM of each primer. For standardization, rat GAPDH was used. The primers for detection of cyclin D1, GluR5–7, and KA1-2 cDNAs were as follows: cyclin D1 (232 bp), 5′-GCGTACCCTGACACCAATCT-3′ and 5′-GCTCCAGAGACAA GAAACGG-3′; GluR5 (208 bp), 5′-GCCCCTCTCACCATCACAT AC-3′ and 5′-ACCTCGCAATCACAAACAGTACA-3′; GluR6 (260 bp) 5′-TTCCTGAATCCTCTCTCCCCT-3′ and 5′-CACCAAATGCCTCC CACTATC-3′; GluR7 (423 bp) were 5′-TGGGCCTTCACCTTGAT CATCA-3′ and 5′-ACTCCACACCCCGACCTTCT-3′; KA1 (267 bp), 5′-GGATCGCTGCTATCTTGGATG-3′ and 5′-CCTTCTCTCCACA GATGTTGCT-3′; KA2 (291 bp), 5′-ACAGCCAGTACGAGACTAC-3′ and 5′-ACTCAGC TTTGGCGCAGAT-3′. PCR conditions were 95 °C for 15 min, followed by 35–40 cycles at 94 °C for 10 s, 49–56 °C for 20 s, and 72 °C for 20 s. After real-time RT-PCR, the reaction products were analyzed by electrophoresis on ethidium bromide, stained agarose gel. All of the PCR experiments were performed in duplicate to verify the results.

Transient forebrain ischemia. Male Wistar rats (8 weeks) were subjected to transient forebrain ischemia by clamping the carotid arteries bilaterally as reported previously [12,13]. Briefly, the animals were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg), and bilateral vertebral arteries were electrocauterized at the level of the first vertebra. On the following day, the common carotid arteries were gently exposed and both arteries were occluded with a vascular clamp for 15 min. The rectal temperature was maintained at 36.5–37.5 °C. Rats that had lost their righting reflexes during the period of ischemia were used as the postischemic group.

Immunohistochemistry. Animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and killed by intracardiac perfusion with isotonic saline followed by a chilled fixative consisting of 4% paraformaldehyde in 0.1 M PB (pH 7.4). After perfusion, the brain was removed and further fixed by immersion in the same fixative overnight at 4 °C, and then immersed in 30% sucrose (pH 7.4) for 72 h at 4 °C. Floating parasagittal sections (30 μm thick) of the hippocampus were prepared by a cryostat (CM1850, Leica, Nussloch, Germany). After blocking with 10% normal goat serum overnight at 4 °C, they were stained with anti-GluR6/7 (10 μg/ml) (Upstate, Lake Placid, NY, USA), anti-CD11b (1: 100) (OX42, Serotec, Bichester, UK) for 3 days at 4 °C. After washing with phosphate-buffered saline (PBS), sections were stained with goat anti-rabbit IgG-conjugated Alexa488 (Jackson Immunoresearch, West Grove, PA, USA) or goat anti-mouse IgG-conjugated Cy3 (Jackson Immunoresearch) for 6 h at 4 °C. The sections were mounted in the anti-fading medium Vectashield (Vector Laboratories, Burlingame, CA, USA) and examined by a confocal laser scanning microscope (CLSM) (LSM510MET, Carl Zeiss, Jena, Germany).

Statistics. The significance of the differences was examined by Student's *t*-test, using Kaleida Graph 3.6 J, and $P < 0.05$ was considered significant.

Results

Density-dependent inhibition of GM-CSF-induced cell proliferation of the GMIR1 cells

Most GMIR1 cells showed an ameboid-like morphology. When the GMIR1 cells were plated at a low cell density in the presence of 0.2 ng/ml GM-CSF, the number of cells markedly increased and reached a confluent state after 7 days in culture (Fig. 1A). The cellular proliferation of the GMIR1 cells was fully dependent on GM-CSF and the number of cells did not change without GM-CSF. The total number of cells increased by approximately 260%, 400%, and 500% after 2, 5, and 7 days in culture, respectively. On the other hand, the ratio of BrdU-incorpo-