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Inhibition of gap junction elevates glutamate uptake in cultured astrocytes 17

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Abbreviations: CNS, central nervous system; Cx, connexin; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; HHBSS, HEPES-buffered Hank's balanced salt solution; HKR, HEPES-buffered Krebs Ringer solution; HRP, horseradish peroxidase; PBS, phosphate-buffered saline.

Abstract—Glutamate uptake is a main function of astrocytes to keep extracellular glutamate levels low and protect neurons against glutamate-induced excitotoxicity. On the other hand, astrocyte networks formed by gap junctions, which are consisted with connexins and connecting neighboring cells, are reported to play a critical role in maintaining the homeostasis in the brain. In the present study, we examined the effects of gap junction inhibitors on the glutamate uptake activity in cultured rat cortical astrocytes. At first, we confirmed the effects of gap junction inhibitors, 1-octanol and carbenoxolone, on cell-cell communication by the scrape-loading assay using a fluorescent dye Lucifer yellow. Both of 1-octanol and carbenoxolone treatments for 20 min in cultured astrocytes significantly suppressed the cell-cell communication assessed as the distance of dye-spreading. 1-octanol and carbenoxolone increased the glutamate uptake by astrocytes and glutamate aspartate transporter (GLAST) expression on the cell membrane. These results suggest that gap junction inhibitors increase the glutamate uptake activity through the increase of GLAST proteins located on the cell membrane. The regulation of gap junction in astrocytes might protect neurons against glutamate-induced excitotoxicity.

Keywords: astrocyte; gap junction; carbenoxolone; 1-octanol; glutamate uptake.

Running Title: Effects of gap junction inhibitors on Glu uptake in astrocytes.

INTRODUCTION

Astrocytes play various important roles in central nervous system (CNS), such as maintenance of blood brain barrier, control of ionic balance in brain parenchyma, cerebrovascular regulation, and scavenging some neurotransmitters including glutamate [1-4]. These functions of astrocytes serve the maintenance of brain homeostasis.

Glutamate is the main excitatory neurotransmitter in the CNS [5]. High concentrations of extracellular glutamate represent a potent neurotoxin which leads to neuronal over-stimulation and subsequent excitotoxic cell death [6]. Astrocytes are known to play complex roles in the control of extracellular glutamate homeostasis [7]. On one hand, astrocytes modulate the duration of glutamatergic neurotransmission and synaptic strength by clearing extracellular glutamate through the high affinity sodium-dependent glutamate transporters, excitatory amino acid transporter-2/glutamate transporter-1 (EAAT-2/GLT-1) and EAAT-1/glutamate aspartate transporter (GLAST) [2, 8, 9]. On the other hand, astrocytes signal to neurons via depolarization- or receptor-induced release of glutamate [7, 10, 11]. Disturbance of GLT-1 and/or GLAST expressions occurs in various brain diseases such as amyotrophic lateral sclerosis, Alzheimer's disease and Huntington's disease, and seems to contribute in part to the neuronal cell death [8].

Gap junction is a cell-cell communication formed by the joining of connexon complexes (also called hemichannels) between neighboring cells and connexon complex is composed of six oligomeric protein subunits called connexins (Cx) [12-15]. Cx is known to have over 20 family proteins and to be highly homologous among various animal species [15]. Gap junctions form closable pores that connect the cytoplasms of neighboring cells, and allow the electric coupling [16] and the diffusion of small molecules up to a molecular weight of approx. 1200 Da such as metal ion, ATP, and second messengers, cAMP and IP_3 [17].

Astrocytes express Cx43, Cx30 and Cx26 [18] and astrocytic networks formed by gap junction regulate extracellular pH, K^+ levels [17, 19] and cause Ca^{2+} wave [20].

In the CNS, it was reported that upregulation of Cx protein might be associated with epilepsy [21, 22] and that the inhibition of gap junction by Cx43 mimetic peptide protected neurons in epileptiform lesion model [23]. Moreover, astrocytic gap junctions composed of Cx43 were reported to be essential for the resistance to oxidative stress [24] and to reduce apoptotic neuronal damage in cerebral ischemia [25]. Taken together, it has been reported that cell-cell communication by gap junction is associated with various diseases and cellular functions; however, the details of the mechanisms how the changes in gap junction regulate cellular functions are unclear.

In the present study, we examined the effects of gap junction inhibitors, 1-octanol and carbenoxolone, on glutamate uptake in cultured rat brain astrocytes. We found that exposure to gap junction inhibitors increased the glutamate uptake activity and GLAST proteins on the cell-surface.

EXPERIMENTAL PROCEDURES

Materials

Deoxyribonuclease I (DNase I; DN-25), trypsin, anti-β-actin antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (whole molecule) antibody were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Dulbecco's modified Eagle medium (DMEM) and horse serum were obtained from Gibco BRL (Grand Island, NY, U.S.A.). Fetal bovine serum was obtained from Biowest (Nuaillé, France). L-[2,3,4-^{3}H]glutamic acid

([^{3}H]Glu) was from American Radiolabeled Chemicals Inc. (St. Louis, MO, U.S.A.). HRP-conjugated goat anti-mouse IgG (H+L) antibody and HRP-conjugated rabbit anti-goat antibody were purchased from Bio-Rad Laboratories Inc. (Hercules, CA, U.S.A.). Pierce BCA Protein Assay Kit and Pierce Cell Surface Protein Isolation Kit were from Thermo Fisher Scientific (Kanagawa, Japan) Carbenoxolone disodium salt was from Aldrich Chemistry (St. Louis, MO, U.S.A.) 1-Octanol was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Lucifer yellow CH dipotassium salt, anti-GLAST antibody, and anti-GLT-1 antibody were purchased from Sigma Life Science (St. Louis, MO, U.S.A.). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Dojindo (Kumamoto, Japan).

Preparation of astrocytes culture

This study was carried out in compliance with the Guideline for Animal Experimentation at Osaka Prefecture University, with an effort to minimize the number of animals used and their suffering. Astrocytes were prepared as described previously [26]. In brief, cortex from 20-day-old embryos, which were taken out from pregnant Wistar rats deeply anesthetized, were cleared of meninges, cut into about 1 mm^3 blocks, and treated with 0.25% trypsin in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS) containing 5.5 mM glucose for 20 min at 37ºC with gentle shaking. An equal volume of horse serum supplemented with 0.1 mg/ml of DNase I was added to the medium to inactivate the trypsin. Then, the tissues were centrifuged at 350 x *g* for 5 min. The tissue sediments were triturated through a pipette with DMEM containing 10% fetal bovine serum, 100 µg/ml streptomycin and 50 unit/ml penicillin. After filtering cell suspensions through a lens-cleaning paper (Fujifilm Co., Tokyo, Japan), the cells were plated on polyethyleneimine-coated 100 mm-diameter plastic dishes (Iwaki, Asahi Glass Co., Tokyo, Japan) at a density of 0.8–1.3 x 10^5 cells/cm^2. Cultures were maintained in a

humidified atmosphere of 5% CO_2 and 95% air at 37ºC with changing medium every 3 days. After one week, astrocytes were replated to remove neurons. On days 12–14, they were replated onto 96-well plates (MS-8096F; for tissue culture, Sumitomo, Tokyo, Japan), 24-well plates (Iwaki), 35 mmm-diameter plastic dishes (Thermo Fisher Scientific), or 60 mm-diameter plastic dishes (Iwaki) using an ordinary trypsin-treatment technique at a density of 1.2 x 10^5 cells/cm^2 and stabilized for 7 days, then we used for experiments.

More than 90% of the cells were immunoreactively positive to an astrocyte marker, glial fibrillary acidic protein; using the antibody (Biosensis Pty Ltd., Thebarton, Australia) and FITC-conjugated anti-mouse IgG antibody. Less than 10% of the cells were positive to a microglial marker, Iba-1; using the antibody (Wako) and rhodamine-conjugated anti-rabbit IgG antibody.

Cell viability

To evaluate cell viability, we measured total mitochondrial activity with so-called MTT assay. In brief, after the cells were stimulated, the medium was changed with a fresh one and one-tenth volume of 5 mg/ml MTT solution was added. The cells were incubated for 1 h at 37ºC and the formazan generated by total mitochondrial activity was dissolved in dimethylsulfoxide, and then the color development was measured at 585 nm with a microplate reader (ARVO 1420 Multilabel counter, Wallac, Turuk, Finland). When we observed cell morphology under a phase-contrast microscope, the remaining cell number is almost consistent with the results of MTT assay.

Cell-cell communication assay

Gap junction-dependent cell-cell communication was assessed by the scrape loading-dye transfer technique as previously reported [27, 28]. Cultured astrocytes were replated on 35

mm dishes and incubated for 7 days. Confluent cells after stimulation were preincubated for 10 min with Ca^{2+} (1 mM)-containing HEPES-buffered Hank's balanced salt solution (HHBSS; 130 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.2) and subsequently rinsed two times with Ca^{2+}-free HHBSS. The cell monolayer was cut with a razor blade (Feather, 0.1 mm thickness) in the presence of Ca^{2+}-free HHBSS containing 0.5 mg/ml Lucifer yellow. After 2 min, cultures were rinsed two times with Ca^{2+}-containing HHBSS and incubated for another 10 min with Ca^{2+}-containing HHBSS. Then, the cells were fixed by 4% paraformaldehyde for 5 min to observe under a fluorescent microscopy. Cell-cell communication was determined as the relative distance of dye-coupled cells far most from the razor incision line in the field of a micrograph (Fig. 1A).

[^{3}H]Glutamate transport assay

Cultured astrocytes were washed with HEPES-buffered Krebs Ringer solution (HKR; 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 50 mM HEPES, and 5.6 mM D-glucose, pH 7.4) twice and subsequent incubation in HKR at 37℃ for 60 min in a 5% CO_2 incubator. The cells were stimulated with gap junction inhibitors for 20 min, and then incubated with 20 μM [^{3}H]Glu at 37℃ for 0 to 40 min. The reaction was terminated by gentle aspiration of the buffer, followed by rinsing with ice-cold HKR containing 1 mM unlabeled Glu at 4℃ three times and subsequent solubilization with 0.1 M NaOH for liquid scintillation spectrometry (LSC-6100, Aloka Co. Ltd., Tokyo, Japan) with 3 ml scintillation cocktail (clear sol I; Nacalai tesque, Kyoto, Japan). Protein concentrations were determined by the method of Bradford using CBB color solution (Nacalai Tesque), according to the manufacturer's protocol, with bovine serum albumin as the standard.

Western blotting

Cultured astrocytes were replated on 60 mm dishes, incubated for 7 days, and then stimulated for 30 min. Cell-surface fraction containing cell membrane proteins was extracted using Pierce Cell Surface Protein Isolation Kit (Thermo Fisher Scienctific), according to the manufacturer's protocol with minor modification. The cells after stimulation were washed with PBS, 0.25 mg/ml EZ-Link® Sulfo-NHS-SS-biotin in ice-cold PBS was added to the cells and treated at 4℃ for 30 min with gentle shaking. Reaction stop solution was added, the cells were collected and centrifuged at 500 x *g* for 3 min. The precipitation was suspended in Tris-buffered saline (25 mM Tris, 150 mM NaCl, pH 7.2) and centrifuged at 500 x *g* for 3 min. Then, the precipitation was homogenized with RIPA buffer (1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% deoxycholic acid, 50 mM NaH_2PO_4, 150 mM NaCl, 2 mM EDTA, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) containing protease inhibitor cocktail (Sigma P8340). The homogenates were sonicated and centrifuged at 10,000 x *g* for 2 min at 4℃. The supernatants were added to Immobilized NeutrAvidin™ Gel to react for 60 min at room temperature with gentle shaking. The reacted solution was centrifuged at 1,000 x *g* for 1 min and the supernatants were collected as "other fraction" which was not biotinylated and not containing cell-surface membrane fraction. On the other hand, the precipitation was resuspended with RIPA buffer containing protease inhibitor cocktail and centrifuged at 1,000 x *g* for 1 min three times. The precipitation was mixed with SDS-PAGE sample buffer (62.5 mM Tris-HCl, 1% sodium dodecyl sulfate, 10% glycerol, pH 6.8) containing 50 mM dithiothreitol for 60 min at room temperature. The mixture was centrifuged at 1,000 x *g* for 2 min and the supernatant was collected as "cell membrane fraction". Protein concentrations were determined by absorbance at 280 nm using NanoDrop ND-1000 (Thermo Fisher Scientific). 0.01% bromophenol blue was added to each sample after the determination of protein concentration, and the samples were stored at −80℃ until use.

"Other fraction" was added at a volume ratio of 4:1 to 50 mM Tris-HCl buffer (pH 6.8) containing 50% glycerol, 10% sodium dodecyl sulfate, 0.05% bromophenol blue and 25% 2-mercaptoethanol, followed by mixing and boiling at 100ºC for 5 min, and stored at −80℃ until use. Protein concentrations were determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), according to the manufacturer's protocol, with bovine serum albumin as the standard.

Each aliquot of a certain amount of proteins was loaded on a 10% polyacrylamide gel for electrophoresis at a constant voltage of 120 V for 2 h at room temperature and subsequent blotting to a polyvinylidene fluoride membrane previously treated with 100% methanol. After blocking by 5% skimmed milk dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween 20, the membrane was reacted with antibodies against GLAST and GLT-1 followed by a reaction with anti-goat or anti-rabbit IgG antibody conjugated with peroxidase. Proteins reactive with those antibodies were detected with the aid of chemiluminescence detection reagents (Immobilon™ Western HRP Substrate; Millipore Corporation, Billerica, MA, U.S.A.) and analyzed with lumino-image-analyzer (LAS-4000, Fujifilm). The graphs showed the density of detection bands.

Data analysis

For statistical analysis of the data, one-way ANOVA followed by Tukey-Kramer multiple comparison procedure or Student's *t*-test was used. Differences between treatments were considered statistically significant when $p < 0.05$.

RESULTS

Effect of gap junction inhibitors on cell-cell communication in cultured astrocytes. Cultured astrocytes were stimulated with various concentrations of gap junction inhibitors, 1-octanol and carbenoxolone for 20 min, and then gap junction-dependent cell-cell communication was confirmed by the scrape-loading assay using Lucifer yellow (Fig. 1A). 1-Octanol significantly suppressed the dye spreading at 0.1-1 mM (Fig. 1B, D). Carbenoxolone also significantly suppressed the dye spreading at 100, 150 μM (Fig. 1C, E).

Both of 1-octanol and carbenoxolone treatment for 20 min used in the present study did not affect the cell viability, although the treatment for 24 h decreased the cell viability assessed by MTT assay (data not shown).

Effect of gap junction inhibitors on glutamate uptake in cultured astrocytes. 20 μM [^{3}H]Glu was added to cultured astrocytes and glutamate uptake for 0-40 min was assayed. Astrocytic glutamate uptake was increased linearly until 10 min and saturated for 30-40 min (Fig. 2A). Therefore, astrocytic glutamate uptake for 10 min was evaluated as the activity henceforth. Cultured astrocytes were stimulated with various concentrations of 1-octanol and carbenoxolone for 20 min, and then glutamate uptake for 10 min was assayed and calculated glutamate uptake per 1 min as the activity. 1-Octanol (0.1-1 mM) increased the glutamate uptake activity in a dose-dependent manner, significantly at 0.3 and 1 mM (Fig. 2B). Carbenoxolone (100, 150 μM) also significantly increased the glutamate uptake activity (Fig. 2C).

Effect of gap junction inhibitors on glutamate transporter on the cell membrane fraction. We assumed to translocate the glutamate transporter expressed on the cell membrane and examined the effect of gap junction inhibitors on expressions of GLAST and GLT-1 proteins.

Cultured astrocytes were stimulated with various concentrations of 1-octanol and carbenoxolone for 30 min, and then GLAST and GLT-1 expressions were assessed by western blotting using “cell membrane fraction” and “other fraction”. GLAST and GLT-1 proteins in “cell membrane fraction” were detected to be very small amounts compared with that in “other fraction”. 1-Octanol (0.1-1 mM) increased GLAST protein on the cell membrane, significantly at 1 mM (Fig. 3A) and that in “other fraction” was not changed (Fig. 3C). Carbenoxolone (100, 150 μM) also significantly increased GLAST protein on the cell membrane (Fig. 3B) and that in “other fraction” was not changed (Fig. 3D).
GLT-1 protein on the cell membrane could not detected (data not shown).

DISCUSSION

In this study, we demonstrated that the glutamate uptake activity and GLAST protein on the cell membrane were increased when the cells were stimulated by gap junction inhibitors, 1-octanol and carbenoxolone, in cultured rat cortex astrocytes. GLAST expression on the cell-surface increased by gap junction inhibitors might be involved in the increase of the glutamate uptake activity. It is likely that gap junction inhibitors increase glutamate uptake by astrocytes *via* cell-surface glutamate transporter and that the increase of glutamate uptake by astrocytes might protect neurons against glutamate-induced excitotoxicity.

We confirmed that gap junction inhibitors, 1-octanol and carbenoxolone, suppressed gap junction-dependent cell-cell communication although the extent of inhibition was less than the previous reports [15, 29]. In the present study, 1-octanol and carbenoxolone stimulations significantly increased the glutamate uptake activity in the cultured astrocytes.

Because the treatment time of gap junction inhibitors to increase the glutamate uptake activity was short as 20 min, we assumed that the gap junction inhibitors did not induce the mRNA and protein expressions of glutamate transporters but changed the location of glutamate transporter proteins from intracellular to cell membrane. In fact, 1-oactanol and carbenoxolone treatments increased GLAST protein levels on the cell membrane; suggesting that the increase of GLAST location on the cell membrane might be involved in the increase of the glutamate uptake activity.

It was reported that 1-octanol bound to cell membrane and carbenoxolone reacted with connexon complex or changed phosphorylation status of connexon complex to inhibit gap junction; however, the mechanisms is unclear [30, 31]. In the cultured astrocytes, gap junction inhibition by 1-octanol and 18α-glycyrrhetinic acid was reported to alter actin organization but not affect glial fibrillary acidic protein and microtubule [32]. Moreover, carbenoxolone, a derivative of glycyrrhetinic acid, is also known to inhibit gap junction [33]. On the other hand, it was reported that glutamate induced rapid upregulation of astrocyte glutamate transport and cell-surface expression of GLAST, and that F-actin organization was involved in the induction [34]. These results suggest that gap junction inhibition by 1-octanol and carbenoxolone might upregulate GLAST transport to cell membrane through the conformational changes of F-actin in the present study. GLT-1 protein on the cell-surface could not detected in the present study; therefore, further investigation is needed.

It was reported that 1-octanol was a lipophilic and affected lipid bilayer on the cell membrane to inhibit Na^+ and K^+ channels other than gap junction [31, 35, 36]. Glutamate transporter is sodium-dependent and conjugated with influx of Na^+ and H^+ and efflux of K^+ [2]. Therefore, in addition to the location of glutamate transporters, the effects of gap junction inhibitors on Na^+ and K^+ channels and Na^+ pump should be further investigated to elucidate the mechanisms of the increase of glutamate uptake by astrocytes.

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Figure legends

Fig. 1 Effects of gap junction inhibitors on cell-cell communication. Assessment of intracellular gap junction coupling by the scrape loading-dye transfer technique. Spreading of the dye was taken as an indicator for intracellular gap junction coupling. Representative schema for the assay is shown in A. Cultured astrocytes were stimulated by various concentrations of 1-octanol (B, D) and carbenoxolone (C, E) for 20 min and assessed the intracellular gap junction coupling. (B, C) Representative photographs are shown. Scale bar = 50 μm. (D, E) The graph shows the distance of the dye spreading. Data are mean ± S.D. of three samples from different cell preparations. $^{**}P < 0.01$, significantly different from control.

Fig. 2 Effects of gap junction inhibitors on glutamate uptake in cultured astrocytes. (A) 20 μM [^{3}H]Glu was added to cultured astrocytes and time-course of glutamate uptake was assayed. (B, C) Cultured astrocytes were stimulated by various concentrations of 1-octanol (B) and carbenoxolone (C) for 20 min. Glu uptake for 10 min was assessed. The graph shows the glutamate uptake activity calculated at control as 100%. Raw data of control = 1.18 ± 0.22 nmol/mg protein/min. Data are mean ± S.D. of four samples from different cell preparations. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control.

Fig. 3 Effects of gap junction inhibitors on GLAST protein on the cell membrane of cultured astrocytes. Cultured astrocytes were stimulated by various concentrations of 1-octanol (A, C) and carbenoxolone (B, D) for 30 min. (A, B) The GLAST protein in “cell membrane fraction” was detected by western blotting. Typical bands of western blotting for GLAST proteins are shown in the photograph. The graph shows the density of detection bands. Data are mean ± S.D. of three samples from different cell preparations. $^{*}P < 0.05$,

significantly different from control. (C, D) The GLAST protein in “other fraction” was detected by western blotting. Typical bands of western blotting for GLAST proteins are shown in the photograph.