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The neuroprotective agent MS-153 stimulates glutamate uptake

Fumiki Shimada [a,b], Yoshio Shiga [b], Maki Morikawa [b], Hiroshi Kawazura [b], Osamu Morikawa [a], Toshiyuki Matsuoka [c], Tomoaki Nishizaki [c], Naoaki Saito [a,*]

[a] *Laboratory of Molecular Pharmacology, Biosignal Research Center, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan*
[b] *Institute of Biological Science, Mitsui Pharmaceuticals, Chiba 297, Japan*
[c] *Department of Physiology, Kobe University School of Medicine, Kobe 650, Japan*

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Abstract

We investigated the effect of (*R*)-(−)-5-methyl-1-nicotinoyl-2-pyrazoline (MS-153), a novel neuroprotective agent, on L-[^{3}H]glutamate uptake through GLT-1, a Na^+/K^+-dependent glial glutamate transporter, expressed in COS-7 cells. MS-153 (1–100 μM) accelerated the L-[^{3}H]glutamate uptake through GLT-1 in a concentration-dependent and time-dependent manner. Eadie–Hofstee analysis revealed that MS-153 significantly decreased the K_m of the glutamate uptake by COS-7 cells expressing GLT-1. In contrast, [^{3}H]gamma-aminobutyric acid (GABA) uptake through a glial GABA transporter was not affected. In addition, MS-153 increased Na^+ currents through GLT-1 expressed in *Xenopus* oocytes. We also investigated the effect of MS-153 on amino acid efflux from rat hippocampal slices. The increase in glutamate efflux induced by 50 mM KCl was significantly attenuated by the treatment with MS-153 at 10 μM, while MS-153 had no significant effect on the K^+-evoked efflux of GABA. Furthermore, the increase in glutamate efflux by ischemia (hypoxia/aglycemia) was partially, but significantly inhibited by MS-153. These results suggest that the cerebroprotective effect of MS-153 in this ischemic model in vivo is due to the specific reduction of the glutamate concentration in the extracellular space, which can probably be attributed to the acceleration of glutamate uptake by the indirect modulation of the glutamate transporter activity.

Keywords: Neuroprotection; Glutamate transporter; Glutamate uptake; Na^+ current

1. Introduction

Glutamate, the major excitatory neurotransmitter in the mammalian central nervous system, has been implicated in the neuronal injury associated with cerebral ischemia (Rothman and Olney, 1986; Meldrum and Garthwaite, 1990). Increased release of glutamate has been observed during both ischemia (hypoxia/anoxia/hypoglycemia/aglycemia) in vitro (Bosley et al., 1983; Mitani et al., 1991) and cerebral ischemia in vivo (Globus et al., 1988). Furthermore, it has been reported that the increase in glutamate release is sustained after occlusive stroke in the brain tissue of a stroke patient (Bullock et al., 1995). Therefore, it is considered that the control of glutamate efflux after ischemia may protect against neuronal cell death by ischemic stroke, although the intracellular mechanisms for ischemic damage are not well elucidated.

It is well known that glutamate efflux into the synaptic cleft by release from nerve endings is counterbalanced by transport into neurons and astrocytes. Glutamate transporters in neurons and glial cells are responsible for the uptake of glutamate released from excitatory nerve terminals (Danbolt, 1994; Gegelashvili and Schousboe, 1997; Robinson and Dowd, 1997). To date, five subtypes of sodium and potassium-dependent glutamate transporters have been cloned and characterized: glutamate transporter (GLT-1) (Pines et al., 1992), glutamate/aspartate transporter (GLAST) (Storck et al., 1992), excitatory amino acid carrier 1 (EAAC1) (Kanai and Hediger, 1992), excitatory amino acid transporter 4 (EAAT4) (Fairman et al., 1995) and excitatory amino acid transporter 5 (EAAT5) (Arriza et al., 1997). Immunocytochemical studies have shown that GLT-1 and GLAST are expressed in astrocytes, while EAAC1 is found both in neurons and in glia and EAAT4 is strictly neuronal (Rothstein et al., 1994; Lehre

* Corresponding author. Tel.: +81-78-803-5961; fax: +81-78-803-5971.
E-mail address: naosaito@kobe-u.ac.jp (N. Saito)

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et al., 1995; Furuta et al., 1997; Conti et al., 1998). In particular, the glutamate transporters in astrocytes play critical roles in maintaining the extracellular concentration of glutamate below the neurotoxic level to protect neurons from glutamate excitotoxicity (Gegelashvili and Schousboe, 1997). This idea has been confirmed recently by the selective genetic knockout of GLT-1 in rodents (Rothstein et al., 1996; Tanaka et al., 1997). In addition, GLT-1 has been associated with the pathogenesis of the neurodegenerative disorder amyotrophic lateral sclerosis (Rothstein et al., 1995).

(*R*)-(−)-5-methyl-1-nicotinoyl-2-pyrazoline (MS-153) is a novel cerebroprotective agent. There is evidence that MS-153 reduces the size of the cerebral infarction that results from middle cerebral artery occlusion in rats (Umemura et al., 1996; Kawazura et al., 1997). MS-153 also reduced brain edema in middle cerebral artery occluded rat (Kawazura et al., 1997). Furthermore, it has been reported that MS-153 suppresses the increase in extracellular glutamate concentration in the ischemic border zone during occlusion of the middle cerebral artery in rat (Umemura et al., 1996). These findings strongly suggest that MS-153 reduces the neuronal damage in the rat focal cerebral ischemia model, although the mechanism of the effect of MS-153 in animal models is unclear.

In the present study, to elucidate the mechanism of the inhibitory effects of MS-153 on the increase in extracellular glutamate concentration in the ischemic rat model (Umemura et al., 1996), we focused on the termination process of glutamatergic neurotransmission and examined the effect of MS-153 on a glial glutamate transporter, GLT-1. Using rat hippocampal slices, the effects of MS-153 on the concentration of extracellular glutamate after stimulation with high K^+ or after ischemia were also investigated.

2. Materials and methods

2.1. Materials

MS-153 was synthesized at the Life Science Laboratory of Mitsui Chemical (Chiba, Japan). Dihydrokainate was purchased from Sigma (St. Louis, MO, USA). MS-153 and DHK were freshly dissolved in distilled water each day. L-[^{3}H]Glutamate (46 Ci/mmol) and [^{3}H]gamma-aminobutyric acid (GABA) (92 Ci/mmol) were obtained from Amersham (Arlington Heights, IL, USA). The full-length GLT-1 cDNA was obtained by screening a rat brain cDNA library with a ^{32}P-labeled synthetic oligonucleotide probe, which was designed based on the sequence of GLT-1 (Pines et al., 1992). The cloned cDNA for GLT-1 was sequenced and found to be identical to that reported previously (Pines et al., 1992). The cDNA encoding GAT-3, a Na^+/K^+-dependent glial GABA transporter, was a gift from Synaptic Pharmaceutical (New Jersey, USA) (Borden et al., 1992). These cDNAs were subcloned into the *Eco*RI site in pBluescript or in pTB701, an expression vector.

2.2. Methods

2.2.1. Cell culture and transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under an atmosphere of 5% CO_2. Cells (5×10^6) were transfected by electroporation (Gene Pulser, Bio Rad, USA) with 32 μg of the expression vector pTB701 containing cDNA of GLT-1 or GAT-3. COS-7 cells transfected by the vector alone were used as the control level of endogenous uptake of L-[^{3}H]glutamate. Transfected cells were divided into 24-well dishes and cultured for 2 days before the uptake assay.

2.2.2. Uptake assay

Two days after transfection, the medium in the dish was replaced by Krebs–Ringer–HEPES solution (KRH) [composition (mM): NaCl 120.0, $MgSO_4$ 1.2, KCl 4.7, KH_2PO_4 2.2, $CaCl_2$ 2.2, glucose 10.0, HEPES 10.0 adjusted with KOH to pH 7.4], and preincubation was done with or without test compound at various concentrations at 37°C for 1–40 min. Glutamate and GABA uptake was initiated by the addition of L-[^{3}H]glutamate or [^{3}H]GABA, respectively. The final concentrations of glutamate and GABA were 2 μM (0.5 Ci/mmol) and 5 μM (0.2 Ci/mmol), respectively. The uptake assay was terminated by washing three times with ice-cold KRH solution. Cells were then solubilized with 1% sodium dodecyl sulfate (SDS), and the radioactivity of the cell extracts was measured with a liquid scintillation counter (LS6500, Beckman, USA). All uptake measurements were performed in duplicate.

2.2.3. In vitro transcription and translation in Xenopus oocytes

mRNAs coding for GLT-1 were synthesized by in vitro transcription. *Xenopus* oocytes were manually separated from the ovary, and incubated overnight in Barth's solution [composition (mM): NaCl 80.0, $MgSO_4$ 0.82, KCl 1.0, $NaHCO_3$ 0.82, $Ca(NO_2)2$ 0.33, $CaCl_2$ 0.41, Tris 7.5 adjusted to pH 7.6] after collagenase (0.5 mg/ml) treatment. Oocytes were injected with GLT-1 mRNAs, and incubated at 18°C.

2.2.4. Voltage-clamp recording

The injected oocytes were transferred to a recording chamber 7 days after incubation and continuously superfused at room temperature (20–22°C) in standard frog Ringer's solution [composition (mM): NaCl 115.0, KCl 2.0, $CaCl_2$ 1.8, HEPES 5.0 adjusted to pH 7.0] or Na^+-free frog Ringer's solution [composition (mM): NaCl 115.0, KCl 2.0, $MgCl_2$ 5.0, HEPES 5.0, EGTA 1.0 adjusted to pH 7.6]. Glutamate-evoked currents were recorded using

two-electrode voltage-clamp techniques with a GeneClamp-500 amplifier (Axon Instruments, USA) and analyzed on a microcomputer using pClamp software (Axon Instruments, version 6.0.3).

2.2.5. Slice preparation

Male Sprague–Dawley rats weighing 250–350 g were used. Each rat was decapitated and the brain was removed quickly and placed into ice-cold Krebs–bicarbonate solution [composition (mM): NaCl 118.0, $MgSO_4$ 1.2, KCl 4.7, $NaHCO_3$ 2.5, KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 10.0, adjusted to pH 7.4 by bubbling continuously with 95% O_2 and 5% CO_2]. Hippocampal slices (400-μm thick) were made using a Rotor Slicer (DTY-8700, Dosaka, Japan) under chilled conditions. The preparations were incubated in the Krebs–bicarbonate solution and equilibrated with 95% O_2, 5% CO_2 for at least 1 h at room temperature.

2.2.6. Superfusion

The preparations were transferred to superfusion chambers (volume 50 mm^3) on a Robotic Perfusion System (Superfusion 2500, Brandel, USA), and superfused dropwise from above at a rate of 0.3 ml/min with oxygenated Krebs–bicarbonate solution at 37°C. The superfusate was collected at intervals during the procedure and frozen immediately. At the end of the experiment, the tissue was placed in 1% SDS in 0.1 M NaOH. The tissue was left at room temperature overnight and then frozen for a later assay of the protein content.

2.2.7. Amino acid efflux by 50 mM KCl

Following a 60-min superfusion with normal Krebs–bicarbonate solution, glutamate efflux was evoked for 1 min by superfusion with Krebs–bicarbonate solution containing 50 mM KCl. In this Krebs–bicarbonate solution containing 50 mM KCl, the concentration of sodium was adjusted to maintain isotonicity. MS-153 (0.01–100 μM) was added in the superfusing solution for 40 min before the K^+ stimulation until the end of each examination. Superfusate samples were collected at intervals of 3 min before and during the stimulation.

2.2.8. Glutamate efflux by ischemia (hypoxia and aglycemia)

Ischemia in vitro was induced by superfusing the slices with the Krebs–bicarbonate solution, in which 10.0 mM glucose was replaced with 10.0 mM sucrose, prebubbled with 95% N_2 and 5% CO_2 for 40 min. After the ischemia, the slices were superfused with normal Krebs–bicarbonate solution for 20 min. O_2 tension in hypoxic solutions was decreased from >500 to 45.3 ± 1.9 mmHg. MS-153 (1 μM) was superfused for 40 min before the ischemia and the same solution containing MS-153 was superfused until the end of each examination. Superfusates were collected at intervals of 3 min. The effects of hypoxia with and without MS-153 were also measured by calculating the area under the curve (AUC) of the glutamate release vs. time plot.

2.2.9. High-performance liquid chromatography (HPLC) analysis

Superfusates were analyzed by HPLC (Burke and Nadler, 1988) after orthophthalaldehyde derivatization of amino acids. Exactly 120 s after derivatization, the reaction mixtures were separated on an ODS-2 5-μm column (4.6×150 mm^2) using 0.1 M sodium phosphate (pH 6.0) containing 35% (v/v) methanol at a flow rate of 1.0 ml/min. The HPLC system consisted of an HPLC pump (655A-11, Hitachi, Japan), a fluorescence detectorimeter (RF-10AXL, Shimazu, Japan) and an integrator (D-2500, Hitachi, Japan). A fluorometer was used for detection of fluorescence at 350/450 nm. Samples were injected automatically by an autosampler (Model 231, Gilson, France). The quantity of glutamate in the superfusates was determined by comparing the area of fluorescence signal to the area of the signal given by a standard. The limit of sensitivity was about 10 fmol. The glutamate efflux from the hippocampal slices was expressed as picomole glutamate efflux per minute per milligram protein. The concentration of protein in the hippocampal slices was determined by using a BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL, USA). The baseline concentration of amino acids was calculated using the prestimulus fraction. Stimulus-induced changes in the levels of glutamate were expressed as a percentage of the baseline levels. Stimulus-induced changes in the concentration of amino acids were compared with those of the vehicle group.

2.2.10. Statistical analysis

The results are expressed as the mean ± S.E.M. The statistical significance of differences between the values was analyzed using either an analysis of variance followed by the Dunnett's test, or by the Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. The effect of MS-153 on the L-[^{3}H]glutamate uptake through the glutamate transporter, GLT-1

The uptake of glutamate by COS-7 cells expressing GLT-1 increased linearly until 3 min of incubation and saturated after 5 min of incubation in the presence of L-[^{3}H]glutamate, and the uptake was saturated when the cells were incubated in more than 100 μM of the extracellular glutamate. In subsequent experiments, the uptake was determined in the presence of 2 μM glutamate for 3 min unless otherwise indicated. The K_m value for the glutamate transporter was 76.2 ± 7.1 μM and the V_{max} value

Table 1
Pharmacological properties of GLT-1 expressed in COS-7 cells
Dihydrokainaite (DHK), L-glutamate and Na^+-free solution were incubated for 40 min. Uptake assays were performed using 2 μM L-[^{3}H]glutamate (0.5 Ci/mmol). Na^+-free KRH solution was prepared by replacing NaCl with an equal molar concentration of choline chloride. Glutamate uptake was expressed as a percentage of the glutamate uptake in the absence of the drug.

	GLT-1 (+)	GLT-1 (−)
	Percentage of control (+)	
Control	100	10
Dihydrokainate (5 mM)	9.2	4.7
(0.5 mM)	25.1	NT
L-Glutamate (1 mM)	2.9	2.6
Na^+-free	1.1	NT

was 5.5 ± 0.37 nmol/min/well. Table 1 shows that the GLT-1 expressed in the present study was Na^+-dependent and dihydrokainate-sensitive, and that its pharmacological properties are similar to those reported (Robinson et al., 1991; Pines et al., 1992). The untransfected COS-7 cells also have GLT-1-like activity, but the activity is significantly lower than that of the transfected COS-7 cells.

Fig. 1 shows the effect of MS-153 on the glutamate uptake by COS-7 cells expressing GLT-1. Pretreatment with MS-153 for 40 min produced a concentration-dependent facilitation of glutamate uptake by these cells, and reached a maximal increase of 26.3% at 10 μM. In contrast, MS-153 failed to enhance the activity of a glial-type GABA transporter, GAT-3, expressed in COS-7 cells (Fig. 1). MS-153 (10 μM) also increased endogenous glutamate transport in untransfected COS-7 cells (data not shown), although the effect of MS-153 was negligible compared with the effect in COS-7 cells expressing GLT-1.

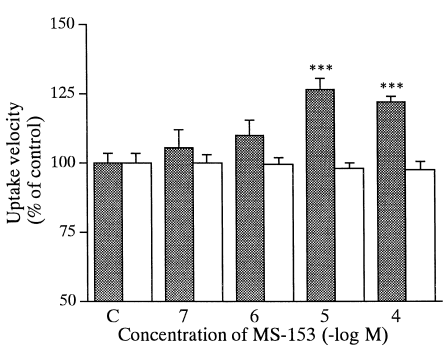

Fig. 1. Effect of MS-153 on glutamate uptake and GABA uptake velocity in COS-7 cells. Uptake assays were performed using 2 μM glutamate, labeled with L-[^{3}H]glutamate tracer (final specific activity, 0.5 Ci/mmol) and 5 μM GABA, labeled with [^{3}H]GABA tracer (final specific activity, 0.2 Ci/mmol). The cells were incubated with MS-153 at various concentrations for 40 min before the application of L-[^{3}H]glutamate and [^{3}H]GABA. Glutamate uptake (hatched columns) and GABA uptake (open columns) were expressed as a percentage of the glutamate and GABA uptake in the absence of the drug. Values are expressed as the mean of five experiments. Vertical bars represent the S.E.M. Asterisks indicate a significant difference from vehicle-treatment: $^{***}P < 0.001$.

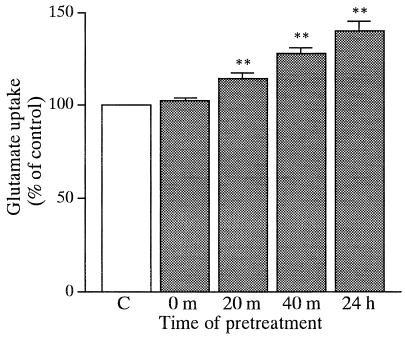

Fig. 2. Effect of MS-153 (10 μM) for various duration of pretreatment on glutamate uptake velocity in COS-7 cells. The duration of pretreatment was at 0, 20, 40 min, or 24 h. The treatment times of L-[^{3}H]glutamate were tested for 3 min. Uptake assays were performed using 2 μM glutamate L-[^{3}H]glutamate tracer (0.5 Ci/mmol). Glutamate uptake was expressed as a percentage of that in the absence of the drug. Values are expressed as the mean of eight experiments. Vertical bars represent the S.E.M. Asterisks indicate a significant difference from vehicle-treatment: $^{**}P < 0.01$.

Simultaneous treatment with MS-153 and initiation of glutamate uptake did not alter the glutamate uptake by COS-7 cells expressing GLT-1. More than 20 min of pretreatment with MS-153 was necessary to induce a significant effect of MS-153 on glutamate uptake (Fig. 2). The enhancement of glutamate uptake by MS-153 was gradually increased when the COS-7 cells expressing GLT-1 were pretreated with MS-153 for longer periods.

The effect of MS-153 on the time-course of glutamate uptake through GLT-1 was further examined. The uptake of glutamate by COS-7 cells expressing GLT-1 was saturated after incubation with L-[^{3}H]glutamate for 15 min in the presence and absence of MS-153 (Fig. 3). The uptake, however, was significantly enhanced by MS-153 when terminated after a short incubation (1, 3 and 5 min) with L-[^{3}H]glutamate. Eadie–Hofstee analysis revealed that MS-

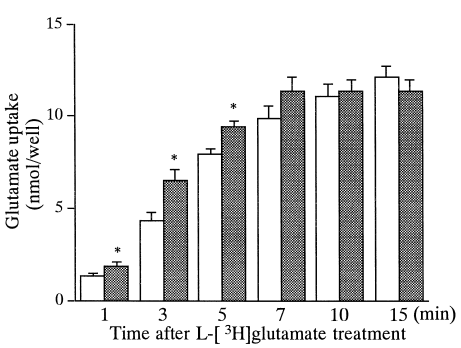

Fig. 3. Effect of MS-153 (10 μM) on the time-course of glutamate uptake by COS-7 cells. The cells were incubated with (hatched columns) or without MS-153 (open columns) for 40 min before the application of L-[^{3}H]glutamate. The treatment times of L-[^{3}H]glutamate were tested for 1, 3, 5, 7, 10, and 15 min. Uptake assays were performed using 2 μM glutamate L-[^{3}H]glutamate tracer (0.5 Ci/mmol). Values are expressed as the mean of five experiments. Vertical bars represent the S.E.M. Asterisks indicate a significant difference from vehicle-treatment: $^{*}P < 0.05$.

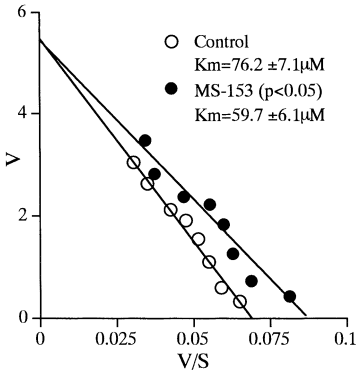

Fig. 4. Eadie–Hofstee analysis of L-glutamate uptake through GLT-1 in the presence and absence of 10 μM MS-153. Uptake velocities were corrected for the nonsaturable component. Values are expressed as the mean of six experiments with S.E.M. $P < 0.05$ (compared with the control by Student's *t*-test).

153 at 10 μM significantly decreased the K_m from 76.2 ± 7.1 μM to 59.7 ± 6.1 μM ($P < 0.05$), while MS-153 had

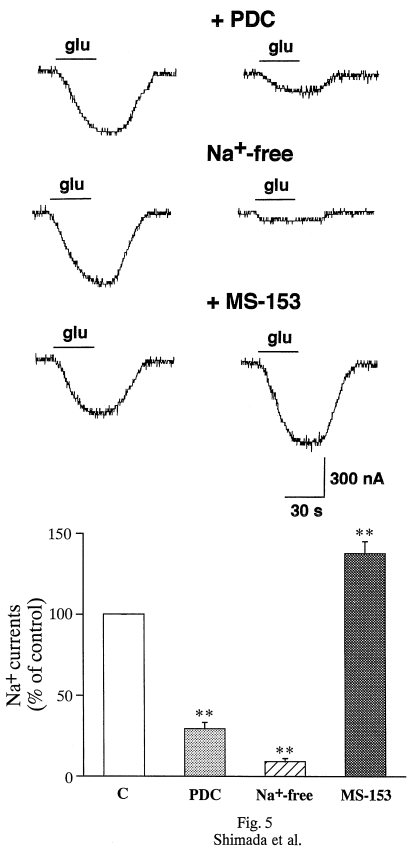

Fig. 5. Effect of MS-153 on Na^+ current in *Xenopus* oocytes expressing GLT-1. Two-electrode voltage-clamps were applied to oocytes expressing GLT-1. Glutamate (10 μM) was bath-applied to a single oocyte before and after 10 min treatment with PDC (300 μM) ($n = 7$) or MS-153 (10 μM) ($n = 14$). In a separate experiment, glutamate-evoked currents were recorded in a medium with replacement of NaCl with LiCl (Na^+-free) ($n = 7$). Typical currents are illustrated in the upper panel. The holding potential was −60 mV. In the accompanying graph, each value represents the mean percentage of control levels. Vertical bars represent the S.E.M. Asterisks indicate a significant difference from vehicle-treatment: $^{**}P < 0.01$.

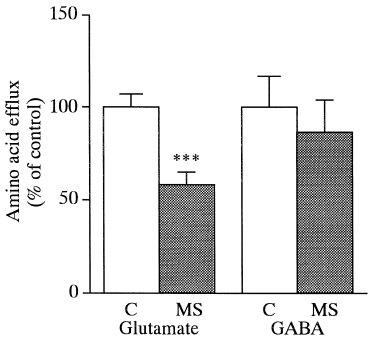

Fig. 6. Effects of MS-153 on 50 mM-KCl-evoked release of glutamate and GABA from rat hippocampal slices. MS-153 at 10 μM was superfused to the slices for 40 min before KCl stimulation. The KCl-evoked release of each amino acid in the presence of MS-153 was expressed as a percentage of the mean values of that without MS-153. The KCl-evoked release of glutamate, but not GABA was inhibited by MS-153. Values are expressed as the mean of 11 experiments. Vertical bars represent the S.E.M. Asterisks indicate a significant difference from vehicle-treated slices: $^{***}P < 0.001$.

no significant effect on the transporter's V_{max} (control, 5.5 ± 0.37 nmol/min/well; MS-153, 5.4 ± 0.25 nmol/min/well) (Fig. 4).

3.2. The effect of MS-153 on Na^+ currents in Xenopus oocytes expressing GLT-1

To examine the effect of MS-153 on glutamate uptake, the glutamate transporter GLT-1 was expressed in *Xenopus* oocytes. One can estimate glutamate uptake by monitoring Na^+ currents, since GLT-1 conveys glutamate in a sodium-dependent manner. In oocytes expressing GLT-1, glutamate generated whole-cell membrane currents, and these currents were inhibited to 29% of control levels by PDC or to 9% of control levels by replacing Na^+ with Li^+ in the extracellular solution (Fig. 5), indicating that GLT-1 actually works in the oocyte expression systems. MS-153 (10 μM) increased currents through GLT-1 to 137% of control levels (Fig. 5), suggesting that MS-153 enhances activity of GLT-1.

3.3. The effects of MS-153 on K^+-evoked amino acid efflux

The effects of MS-153 on the neurotransmitter efflux, especially on amino acid efflux, were examined. We measured the spontaneous efflux of two amino acids (glutamate and GABA) from the hippocampal slices. Spontaneous release of glutamate was 12.8 ± 1.3 pmol/min/mg protein, but the spontaneous efflux of GABA was too low to be detected. Then, we examined the effects of MS-153 on K^+-evoked efflux of the two amino acids. In the presence of 50 mM KCl for 30 s in the superfusion medium, significant increase in the release of glutamate (51.8 ± 5.8 pmol/min/mg protein) was observed. GABA efflux was also increased by 50 mM KCl from an unde-

tectable level to 45.7 ± 6.9 pmol/min/mg protein. The K^+-evoked efflux was observed in the superfusate during K^+ stimulation (3 min), then disappeared in the following superfusates. Fig. 6 shows the effects of MS-153 on K^+-evoked efflux of glutamate and GABA from rat hippocampal slices. MS-153 at 10 μM significantly attenuated the K^+-evoked glutamate efflux by 58.0%. On the other hand, MS-153 (10 μM) had no significant effect on the K^+-evoked efflux of GABA. The inhibitory effect of MS-153 on glutamate efflux was observed when lower doses of MS-153 (0.1 and 1 μM) were used (data not shown). The attenuation of glutamate efflux by MS-153 was partial, and MS-153 did not completely abolish the K^+-evoked glutamate efflux even at 10 μM or higher (data not shown). However, the spontaneous glutamate efflux was not influenced by the treatment with MS-153 for 40 min at 10 μM (data not shown).

3.4. The effect of MS-153 on the glutamate efflux evoked by ischemia (hypoxia and aglycemia) in vitro

Fig. 7 represents the effect of MS-153 at 1 μM on the glutamate efflux by ischemia for 40 min. The glutamate efflux was markedly increased by hypoxia and aglycemia, peaking at 15 min (900 ± 110% of the spontaneous release) after the beginning of the treatment. Thereafter, the increase in glutamate efflux by ischemia rapidly declined. MS-153 (1 μM) significantly inhibited the amount of glutamate efflux by ischemia, while the time course of glutamate efflux was not affected. The peak value of glutamate efflux in the presence of MS-153 was inhibited to 410 ± 70% of the spontaneous efflux. The AUC (percentage of the spontaneous efflux for 40 min.) was significantly inhibited from 10400 ± 1700% (control) to 3800 ± 810% (MS-153) (Fig. 7 inset).

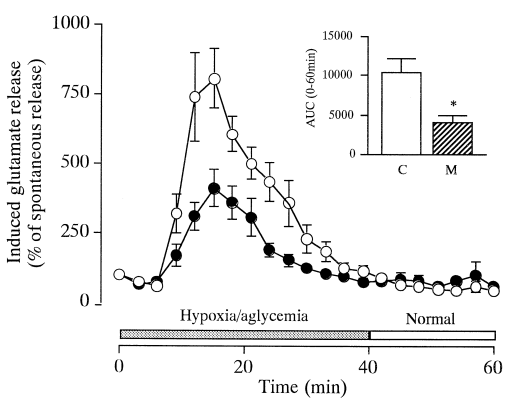

Fig. 7. Effects of MS-153 on glutamate release during hypoxia from rat hippocampal slices. MS-153 (1 μM) was superfused from 40 min before the treatment of hypoxia and aglycemia. The release of glutamate induced by hypoxia and aglycemia was partially blocked by the treatment with MS-153. Values are expressed as the mean of seven experiments. Glutamate release was expressed as a percentage of the release before the hypoxic treatment. The inset shows the area under the curve (AUC) of the glutamate release vs. time plot. The AUCs were calculated from an increase in the glutamate release for 40 min after the onset of hypoxia. Vertical bars represent the S.E.M. Asterisks indicate a significant difference from vehicle-treated slices: $*P < 0.05$.

4. Discussion

We investigated the effect of MS-153 on L-[^{3}H] glutamate uptake using COS-7 cells expressing GLT-1. MS-153 enhanced the uptake of glutamate by these cells in a concentration-dependent manner. Eadie–Hofstee analysis showed that the effect of MS-153 on GLT-1 is mainly due to the increase in the affinity of GLT-1 for extracellular glutamate. Na^+-dependent GABA transport through GAT-3 was not altered by MS-153, indicating that MS-153 does not influence the electrochemical gradient of sodium ions in the cells. We also investigated the effect of MS-153 on Na^+ currents through GLT-1 in *Xenopus* oocytes. MS-153 significantly increased Na^+ currents through GLT-1, suggesting that MS-153 enhances the activity of GLT-1. In addition, MS-153 did not affect the activity of the Na^+/K^+-ATPase (data not shown), which relates to the sodium/potassium electrochemical gradient as a driving force of the glutamate transporter. From the finding that MS-153 could not enhance GLT-1 activity without substantial pretreatment of the cells with MS-153, it is suggested that MS-153 does not act on the extracellular region of GLT-1 directly, but rather on the intracellular regions of GLT-1 after permeation into the cell. As MS-153 is not structurally related to dihydrokainate or other glutamate analogues, MS-153 does not appear to act on GLT-1 as a competitor with endogenous glutamate. The effect of MS-153 on GLT-1 may be mediated by yet unknown intracellular proteins that modulate the GLT-1 activity. The activity of GLT-1 is regulated by its phosphorylation by protein kinase C (Casado et al., 1993; Ganel and Crosson, 1998), and MS-153 inhibits the translocation of protein kinase C induced by transient ischemia in hippocampal slices (Sakata et al., 1997). It is possible that MS-153 facilitates the GLT-1 activity by the modulation of the PKC pathway, although the mechanism remains to be clarified.

The facilitating effect of MS-153 on glutamate uptake strongly suggests that MS-153 decreases the amount of extracellular glutamate in the synapse. Because GLT-1 is abundantly expressed in the hippocampus (Rothstein et al., 1994; Lehre et al., 1995; Furuta et al., 1997), we examined the effect of MS-153 on the amount of extracellular glutamate (glutamate efflux) after K^+-stimulation using the hippocampal slices. As shown in Fig. 6, MS-153 at 10 μM decreased the K^+-stimulated efflux of glutamate, although the efflux of GABA was not influenced by MS-153. The effect of MS-153 specific for glutamate efflux agreed with the present findings that MS-153 acts on a glutamate transporter, but not on a GABA transporter. As the spontaneous glutamate efflux was not altered by MS-153, it is

suggested that glutamate uptake through GLT-1 does not play an important role in maintaining the spontaneous level of glutamate and that MS-153 specifically inhibits glutamate efflux only when the hippocampal cells are excited by depolarization.

The excessive accumulation of glutamate in the extracellular space results in neuronal death (Choi et al., 1987) and is thought to be involved in ischemic brain damage (Rothman and Olney, 1986; Meldrum and Garthwaite, 1990). It is considered that the neuronal toxicity of glutamate is due to an excessive and sustained activation of glutamate receptors. To date, inhibition of glutamate toxicity, such as suppression of glutamate release (Wahl et al., 1993; Bullock et al., 1995), antagonism of glutamate receptors (Hatfield et al., 1992; Graham et al., 1996), or blocking of various Ca^{2+} channels (Takakura et al., 1991; Valentino et al., 1993), has been proposed as a therapeutic intervention for ischemic damage. We have recently reported that MS-153 has a neuroprotective effect after ischemia, such as the reduction in size of cerebral infarctions and the improvement in neurological deficits induced by middle cerebral artery occlusion in rats (Umemura et al., 1996; Kawazura et al., 1997). The enhancing effect of MS-153 on GLT-1 activity and the inhibitory effect of MS-153 on glutamate efflux strongly suggests that the neuroprotective action of MS-153 in the rat ischemic model is due to inhibition of glutamate transmission in the synaptic cleft. We also studied the effect of MS-153 on glutamate efflux by ischemia (hypoxia/aglycemia). Hypoxia is known to alter, to some extent, the metabolism of a number of neurotransmitters such as monoamines and amino acids (Siesjö, 1978). Transmitter release is produced by an energy-requiring biochemical process that is sensitive to the reduced ATP yield from glucose oxidation that occurs during hypoxia (Tower, 1979). As shown in Fig. 7, MS-153 at 1 μM significantly decreased the glutamate efflux by hypoxia. Matsuda et al. (1992) reported that hypoxia-induced glutamate efflux is produced by an inhibition of Na^+/K^+-ATPase and Ca^{2+}-ATPase activity which would result from a decrease in ATP content and by the elevation of intracellular Ca^{2+} concentration. It is possible that MS-153 inhibits the hypoxia-induced glutamate efflux by acting on Ca^{2+} channels, although MS-153 did not inhibit Ca^{2+} channels in preliminary experiments (data not shown). Furthermore, under ischemic conditions, glutamate was released through the glutamate transporter by a reversed uptake mechanism from neurons and astrocytes, and the extracellular glutamate increased to levels high enough to be excitotoxic (Nicholls and Attwell, 1990; Szatkowski and Attwell, 1994). From the present results that the hypoxia-induced increase in extracellular glutamate through reversed uptake was reduced by MS-153, MS-153 does not appear to enhance the reversed uptake which is induced by hypoxia and possibly suppresses the reversed uptake. However, the effect of MS-153 on reversed uptake should be further investigated. It also has been reported that the expression of GLT-1 is reduced after global transient ischemia (Torp et al., 1995). These results strongly suggest that glutamate uptake through the glutamate transporter plays a protective role in brain ischemia. Furthermore, the neuroprotective effects of MS-153 after ischemia are due to the specific reduction in the amount of extracellular glutamate by the facilitating effect of MS-153 on the glutamate uptake through glutamate transporters.

In conclusion, we demonstrate that MS-153 enhances the GLT-1 activity and inhibits the depolarization- and ischemia-induced efflux of glutamate, but not of other amino acids. These findings confirm that MS-153 is a potential therapeutic agent in ischemic stroke.

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