Laboratory Investigations

Midazolam and ketamine inhibit glutamate release via a cloned human brain glutamate transporter

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Purpose: In cerebral ischemia/anoxia, the glutamate transporter runs in reverse and releases glutamate into the extracellular space, causing irreversible neuronal damage. Intravenous anesthetics attenuate overall glutamate release and prevent neuronal injury during anoxia/ischemia, but their effect on the glutamate transporter is variable.

Methods: A human glial glutamate transporter (hGLT-1) cDNA was isolated by screening a human cerebral cortical library. Cloned cDNA was transfected in Chinese hamster ovary cells. The effect of the intravenous anesthetics midazolam (0.3 to 30 μ M), ketamine (10 to 100 μ M), thiopental (30 to 300 μ M), and propofol (3 to 30 μ M) on reversed uptake of L-glutamate via hGLT-1 was examined by whole-cell patch-clamp.

Results: Midazolam at a concentration 3 μ M reduced outward currents arising from reversed L-glutamate uptake via hGLT-1 in a concentration-dependent manner. While, ketamine at 100 μ M attenuated the same outward currents, to $53.3 \pm 11.4\%$ of those seen in controls without anesthetics ($P < 0.05$, $n=5$). In contrast, neither thiopental nor propofol showed effects on outward currents mediated by reversed operation of hGLT-1.

Conclusions: These results suggest that midazolam and ketamine, but not thiopental and propofol, have a capacity to inhibit glutamate release via GLT-1 directly.

Objectif : Lors d'ischémie/anoxie cérébrale, l'action du transporteur de glutamate est inversée, ce qui libère du glutamate dans le compartiment extracellulaire et cause des dommages neuronaux irréversibles. Les anesthésiques intraveineux réduisent la libération totale de glutamate et préviennent les lésions neuronales pendant l'anoxie/ischémie, mais leur effet varie sur le transporteur de glutamate.

Méthode : Un transporteur glial de glutamate humain (TGLh-1) ADNc a été isolé à la suite d'une sélection dans la génothèque corticale cérébrale humaine. L'ADNc cloné a été transféré dans les cellules ovariennes d'un hamster chinois. L'effet des anesthésiques intraveineux midazolam (0,3 à 30 μ M), kétamine (10 à 100 μ M), thiopental (30 à 300 μ M), et propofol (3 à 30 μ M) sur la fixation inversée de L-glutamate au moyen du TGLh-1 a été examiné selon la technique de «patch-clamp» d'une cellule complète.

Résultats : Le midazolam, à 3 μ M, a réduit d'une manière dépendante de la concentration le courant sortant provenant de la fixation inversée de L-glutamate au moyen du TGLh-1. La kétamine, à 100 μ M, a diminué le même courant sortant à $53,3 \pm 11,4\%$ des témoins sans anesthésique ($P < 0,05$, $n=5$). Par ailleurs, ni le thiopental ni le propofol n'ont montré d'effets sur le courant sortant dont l'origine serait attribuée à l'opération inversée du TGLh-1.

Conclusion : Ces résultats suggèrent que le midazolam et la kétamine, non le thiopental ni le propofol, ont la capacité d'inhiber la libération de glutamate en passant directement par le TGL-1.

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Accepted for publication February 6, 2000.

DURING cerebral anoxia and ischemia, glutamate accumulates in the extracellular space of the brain at concentrations that hyperexcite glutamate receptors to result in uncontrolled calcium influx through the N-methyl-D-aspartate (NMDA) receptor; consequent Ca^{2+} -mediated damage leads to cell death.^{1,2} The chief source of extracellular glutamate accumulation during ischemia is failure or reversal of glutamate transporters rather than synaptic vesicular release of glutamate.³ Glutamate transporters, widely distributed in the brain and spinal cord, maintain extracellular glutamate concentrations below neurotoxic levels.⁴⁻⁶ However, in cases where electrochemical ionic gradients are disrupted, such as during brain ischemia and anoxia, glutamate transporters run in the reverse, thereby depositing glutamate into the extracellular space.^{3,7,8} Uptake and reversed uptake of glutamate via glutamate transporter exhibits a fixed electrogenic stoichiometry.⁹⁻¹² (Figure 1).

In recent years, five homologous types of glutamate transporter, termed GLAST, GLT-1, EAAC1, EAAT4 and EAAT5, have been cloned from various species.¹³⁻¹⁷ The GLAST transporter is found in both neurons and astroglia, while EAAC1 is specific to neurons.⁴ The EAAT4 transporter is expressed predominantly in the cerebellum¹⁶ and EAAT5 has been identified in the retina.¹⁷ The GLT-1 transporter represents the predominant glial transporter in the cerebrum.⁴ GLT-1 is the most abundant glutamate transporter in the brain, and is an important effector of clearance of free glutamate from the synaptic cleft.^{5,18} Whereas, the GLT-1 transporter may contribute to a component of glutamate release during anoxia/ischemia, because glutamate release from glial cells has been demonstrated under pathophysiological conditions.^{3,8,19-21}

It has been argued for sometime that general anesthetics protect cerebral ischemia, however, the mechanisms by which they protect neurons and other cells in the central nervous system have not been clearly established. Although a few intravenous anesthetics attenuate elevation of the extracellular glutamate concentration during anoxia/ischemia,²²⁻²⁶ these previous studies on glutamate release have been based on complex preparations such as tissue slices,^{2,3} synaptosomes,^{2,6} and *in vivo* whole-brain,^{22,24,25} which are likely to reflect activities of multiple transporter subtypes, receptors, ion channels and pumps. Cloning of glutamate transporter cDNAs offers a unique opportunity for independent investigation of individual members of this family after expression in a heterologous system.²⁷ We, therefore, constructed cell lines that express, in a stable fashion, a cloned human glial

glutamate transporter (hGLT-1). The goal of this study was to examine the effects of the intravenous anesthetics midazolam, ketamine, thiopental, and propofol on glutamate release via hGLT1 using whole-cell patch-clamp.^{2,8}

Methods

Cloning of a cDNA encoding hGLT-1

A GLT-1 cDNA (1.7 kbp) clone was isolated by the polymerase chain reaction (PCR) from an adult human cerebral cortex cDNA library (Clontech, Palo Alto, CA). PCR including denaturation (one minute, 94°C), annealing (two minutes, 55°C), and extension (three minutes, 72°C) for 35 cycles, followed by further incubation for seven minutes at 72°C, was performed in 50 μL of reaction mixtures that contained oligonucleotide primers at 0.3 μM each, the adult human cerebral cDNA library, 200 μM of each deoxynucleotide, and Vent DNA polymerase five units (NEB, Beverly, MA). Two primers, 5'-AGACCATGGCATCTACGGAA-3' and 5'-TTAGCATAGGATACGCTGGG-3' submitted to the GenBank/EMBL/DDBJ Data Bank,²⁹ were designed. PCR products were subcloned into a pUC18 vector (Stratagene, La Jolla, CA) after digestion with Sma I, and were amplified by transformation of Epicurian Coli XL1-Blue MRF'Kan supercompetent cells (Stratagene). The hGLT-1 cDNA was ligated into a eukaryotic expression vector (pcDNA3.1); this plasmid was transfected into CHO cells using the calcium phosphate method.³⁰ Clonal cell lines expressing the highest level of hGLT-1 were selected for subsequent experiments.

FIGURE 1 “Normal” glutamate uptake (a) and reversed glutamate uptake (b). (a) Under normal conditions, the uptake of one glutamate anion mediated by glutamate transporter is accompanied by the co-transport of two or three Na^+ ions into the cell, and by the countertransport of one K^+ ion out of the cell, and either co-transport of one H^+ ion or countertransport of one OH^- ion, resulting in the movement of a few positive charges inward per glutamate anion entering the cell. (b) The reversed uptake shows that two or three Na^+ ions and one glutamate anion leave the cell while one K^+ ion and one OH^- ion enter or one H^+ leaves, leading to an outward current from the release of one or two positive ionic charges. This electrogenic stoichiometry facilitates investigation of the ionic movements driving the glutamate transporter.

FIGURE 2 Effect of midazolam on K⁺-evoked outward current due to the reversed uptake of L-glutamate via the human glutamate transporter hGLT-1. The current elicited by raising the extracellular K⁺ concentration, [K⁺]_o, from 0 to 10 mM outside CHO cells expressing hGLT-1 that were clamped to 0 mV with a pipette containing 10 mM Na⁺ and L-glutamate was measured in the presence of various concentrations of midazolam. The data points defining the dose-response curve for the effect of midazolam on K⁺-evoked outward current are expressed as percentages of the control value before exposure to midazolam. Each point shows data from 3 to 13 cells. Midazolam decreased K⁺-evoked outward currents in a concentration-dependent manner (top). A chart-recorder tracing of reversed L-glutamate uptake current via hGLT-1 obtained from one cell under control conditions, during application of midazolam (1 and 10 μM), and after washout for 10 min in drug-free bath solution, is shown where [K⁺]_o was raised (black bars, 30 sec) from 0 to 10 mM at a holding potential of 0 mV (bottom)

Cell growth and expression

Unaltered (intact) CHO cells and stable transfected cells with hGLT-1 were cultured in Dulbecco's modified Eagle's medium (D-MEM); (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 units·mL⁻¹ penicillin G, 100 μg·mL⁻¹ streptomycin, and 250 ng·mL⁻¹ amphotericin B in a CO₂ 0.5% atmosphere in a humidified incubator at 37°C. Transfected CHO cells were selected using 600 μg·mL⁻¹ of Geneticin (Gibco-BRL). Cells were grown in 24-well plates, each well containing about 10⁴ to 10⁵ cells was used for electrophysiological experiments two to three days after plating, before the cell layer became confluent.

Reversed uptake of L-glutamate by hGLT-1

The whole-cell patch-clamp was used to record membrane currents using Axopatch 1D (Axon Instruments, Foster City, CA). The tip resistance of the suction pipette filled with the pipette solution was 2 to 5 M. Currents were filtered at 1 kHz, digitized, recorded to

FIGURE 3 A typical chart recorder tracing of K⁺-evoked outward current due to reversed uptake of L-glutamate when ketamine, thiopental and propofol was applied intermittently to CHO cell expressing hGLT-1 at 0 mV. The extracellular K⁺ concentration, [K⁺]_o, was raised from 0 to 10 mM (black bars, 30 sec). Exposure to ketamine at concentrations greater than a 30 μM marked in reduced K⁺-evoked outward current. In contrast, administration of thiopental (100 and 300 μM) or propofol (3 to 30 μM) exerted no observable effect on K⁺-induced outward current.

thermal array recorder (Nihon Kohden, Tokyo, Japan), and analyzed using pClamp software (Axon Instruments).

Cells attached to culture dishes were mounted on the stage of an inverted microscope and perfused with Na⁺- and K⁺-free external solution in which NaCl was replaced with choline chloride contained concentrations (mM) of: choline chloride 110, MgCl₂ 0.5, CaCl₂ 3, HEPES 5, glucose 15, BaCl₂ 6, and ouabain 0.1, pH was adjusted to 7.4 with N-methyl-D-glucamine (NMDG). Barium was included to block most of the K⁺ conductance of the cell. The Na⁺-K⁺ exchanger was inhibited with 0.1 mM ouabain. Pipette solution constituents had the following concentrations (mM): Na glutamate 10, choline chloride 92, (NMDG)₂-EGTA 5, HEPES 5, CaCl₂ 1, MgCl₂ 2, MgATP 5, and NMDG to attain a pH of 7.0. Based on the stoichiometry of the glutamate transporter (Figure 1), cells were whole-cell voltage-clamped at -40 mV of holding potential and initially superfused with Na⁺- and K⁺-free external solution to prevent the reverse uptake current. Next, by application of the K⁺-including external solution contained concentrations (mM) of: choline chloride 100, KCl 10, MgCl₂ 0.5, CaCl₂ 3, HEPES 5, glucose 15, BaCl₂ 6, and ouabain 0.1,

pH was adjusted to 7.4 with N-methyl-D-glucamine (NMDG), K⁺-induced outward currents were recorded at 0 mV. The current-voltage relationship was obtained by external application of KCl during depolarizing pulses to potentials between -80 and +60 mV in 20 mV increments from -40 mV of a holding potential. After control data were recorded, each test solution of midazolam, ketamine, thiopental, and propofol was applied externally to the CHO cells for three minutes, and K⁺-evoked outward currents were measured for 30 sec at 0 mV. The recovery data were measured for 10 min, the data which did not show recovery rate of more than 50% were discarded. Temperature on external solution was monitored with a thermometer. All experiments were performed at 34 to 35°C.

Materials

Midazolam (Yamanouchi, Tokyo, Japan), thiopental (Tanabe, Osaka, Japan), and ketamine (Sankyo, Tokyo, Japan) were prepared fresh before every experiment. Propofol was a gift from Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). Dimethyl sulfoxide (DMSO) was used as a vehicle for propofol at a final concentration of 0.1% (v/v). Each agent was diluted in Na⁺-free external solution to obtain the following final concentrations: midazolam, 0.3 to 30 μ M; ketamine, 10 to 100 μ M; thiopental, 30 to 300 μ M; and propofol, 3 to 30 μ M. These test solutions were adjusted to pH 7.4 with NMDG. All anesthetic concentrations indicated in this report are expressed as free concentrations in aqueous solution.

Statistical analysis

All data are expressed as means $\pm$ SEM. For statistical analysis, one-way ANOVA with post hoc Scheffé F test was used. Differences were considered statistically significant when P was < 0.05 .

Results

When extracellular concentration of K⁺ was increased from 0 to 10 mM at 0 mV, outward currents were evoked in CHO cells expressing hGLT-1 (200.4 ± 21.7 pA, $n=25$), but not in unaltered CHO cells ($n=8$, data not shown). Using glutamate-free internal solution, K⁺-evoked outward currents were not observed even in CHO cells expressing hGLT-1 ($n=5$, data not shown). Application of midazolam at concentrations > 3 μ M decreased K⁺-evoked outward currents in a concentration-dependent manner (Figure 2). Exposure to ketamine at 30 μ M and 100 μ M concentrations reduced the corresponding current to $88.5 \pm 5.1\%$ (NS, $n=5$) and $53.3 \pm 11.4\%$ of control ($P < 0.05$, $n=5$), respectively. In contrast, administration of thiopental

($n=5$) or propofol ($n=5$) exerted no measurable effect on K⁺-evoked outward current. After washout of midazolam and ketamine, K⁺-evoked outward currents recovered to $85.0 \pm 3.8\%$ ($n=21$) and $77.2 \pm 10.1\%$ ($n=5$) of the control, respectively.

Discussion

The present study demonstrated that midazolam at concentrations above 3 μ M attenuated K⁺-evoked outward current in a concentration-dependent manner, and that ketamine at a concentration of 100 μ M decreased the same outward current significantly. In contrast, thiopental and propofol had no observable effect on K⁺-induced outward current. In a previous study using the same cell lines,³¹ we confirmed that the electrophysiological and pharmacological properties of hGLT-1 expressed in CHO cells were similar to those found in glial (Müller) cells,³² and that K⁺-evoked outward current in a specific experimental condition, mimicking anoxia/ischemia by significantly modifying the ion gradient, resulted from the reversed operation of hGLT-1. These results suggest that midazolam and ketamine, but not thiopental and propofol, directly inhibit reversal of L-glutamate uptake via hGLT-1.

Midazolam, a benzodiazepine, binds with high affinity to peripheral-type benzodiazepine receptors³³ in several different tissues including liver, lung, kidney, heart, adrenal gland, testis, ovary, erythrocyte, smooth muscle, and brain. In the brain, peripheral-type benzodiazepine receptors are limited to non-neuronal cells, mainly astrocytes.³⁴ Astrocytes express peripheral-type benzodiazepine receptors but not central-type benzodiazepine receptors.³⁵ In contrast to central-type benzodiazepine receptors, which are linked to the GABA receptor-chloride channel complex, peripheral-type benzodiazepine receptors in brain tissue are linked to voltage-dependent Ca²⁺ channels, especially the L-type, where they inhibit channel activity.³⁶ Because the actions of midazolam demonstrated in the present study were independent of Ca²⁺, the effect of midazolam on the reversed uptake of L-glutamate via hGLT-1 may have a novel mechanism not involving benzodiazepine receptors.

Several investigators have reported that ketamine can protect the brain against ischemic injury.^{37,38} In patients anesthetized with ketamine, plasma concentrations have been reported to be approximately 5 to 10 μ M,^{30,40} but brain concentration of ketamine may be considerably higher because the drug is lipophilic. Inhibition of reversed operation of hGLT-1 by ketamine is a likely explanation for the neuroprotective effect of this agent. The form of ketamine used in the present study was a racemic mixture consisting of two

optical enantiomers, R (-) and S (+). Further studies using the R (-) or S (+) enantiomer should help to differentiate a pharmacological effect from a nonspecific toxic effect.⁴¹

In the current study, we could find no evidence of decreased glutamate release from thiopental-treated cells. The absence of effect is consistent with previous findings that pentobarbital did not affect extracellular glutamate concentration measured *in vivo* by microdialysis in the dorsal hippocampus of the rabbit.²² This lack of effect supports observations that barbiturates are not effective in ameliorating neurological injury associated with severe global cerebral ischemia.⁴² Pentobarbital prolongs only the time to terminal depolarization under anoxic/ischemic conditions; it does not affect the rate at which the extracellular glutamate concentration increases.²⁵ Neuroprotective effects of barbiturates may be explained by mechanism other than inhibition of reversed operation of GLT-1 during anoxia/ischemia.

A highly lipophilic compound such as propofol can be expected to have multiple actions on excitable membranes. In the present study, however, propofol failed to attenuate K⁺-evoked outward current due to reversed uptake of L-glutamate via hGLT-1. Previous studies had reported that propofol did not affect hypoxia-evoked glutamate release from rat cortical brain slices,²³ and did not reduce the neuronal damage in rats subjected to focal ischemia.⁴³ In addition, propofol had no measurable effect on reversed uptake by the glutamate transporter in the rabbit dorsal hippocampus²² and had no direct effects on the Na⁺-dependent glutamate transporter in rat cerebrocortical synaptosomes.²⁶ These previous studies and our result indicate that propofol has little or no direct effect on glutamate release via the glutamate transporter.

In examining the limitations of this study, first we must consider the probability that hGLT-1 functions differently in CHO cells *in vitro* vs glial cells *in vivo*. Our electrophysiological experiments required specific intracellular and extracellular solutions, characterized by replacement of intracellular contents with a zero-K⁺ solution, removal of Na⁺ from external solution, inhibition of Na⁺/K⁺ ATPase with ouabain, blockade of K⁺ conductance with barium, and suppression of changes in pH and ATP. The pipette solution dialyzes the cell cytoplasm so that, over the course of time, the cytosolic concentrations of ions equal to those in the pipette. Moreover, we measured glutamate efflux into a substrate-free solution driven by a virtually infinite outward gradient.²¹ Therefore, similar effects may vary with other simulations of anoxia/ischemia. Secondly, this study deals with only one of at least five transporters for

glutamate transfer. Effects demonstrated in one cloned transporter (hGLT-1) cannot translate to other glutamate transporters such as EAAC1. Thirdly, the concentrations of midazolam and ketamine where an effect was found are orders of magnitude greater than clinical serum concentrations. The concentrations of midazolam required in the current study were well above those required to saturate the benzodiazepine binding sites on astrocytes, as indicated by an IC₅₀ of 106 nM to displace midazolam bound to astrocytes in primary culture.³⁵ Therefore, a nonspecific mechanism related to the high lipid solubility of midazolam at a physiologic pH cannot be excluded. Further studies are necessary to justify direct effects of midazolam and ketamine on glutamate transporters.

In conclusion, midazolam and ketamine directly attenuated the reversed uptake of L-glutamate via hGLT-1 under non-physiologic ionic conditions, while thiopental and propofol had little or no effect. The neuroprotective effects of midazolam and ketamine observed during anoxia/ischemia may be attributed in part to inhibition of reversed uptake of L-glutamate via GLT-1.

Acknowledgments

The authors wish to express their gratitude to Kohei Sawada PhD, Toshihide Hashimoto PhD, Tsukuba Research Laboratories, Eisai Co., Ltd., Tsukuba, Ibaraki, Japan, for their technical assistance and valuable comments in preparing the manuscript.

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