GABA-Mediated Inhibition of Glutamate Release During Ischemia in Substantia Gelatinosa of the Adult Rat

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INTRODUCTION

Ischemic stroke is frequently encountered following vascular disorders or clinical practices, resulting in the death of CNS neurons (see Dinnage et al. 1999 for review). There is much evidence that ischemia causes an excess of an excitatory neurotransmitter, L-glutamate, at synapses in the CNS including the spinal cord (Marsala et al. 1995), which triggers the death of CNS neurons through its depolarizing action (see Choi and Rothman 1990 for review). To prevent such an excitotoxicity, CNS neurons are endowed with a protective system occurring in a pre- and postsynaptic manner. For example, there is a presynaptic inhibition in glutamatergic transmission or a membrane hyperpolarization following ischemia. The former action in the rat hippocampal CA1 region is induced by adenosine produced as a result of ischemia (Coelho et al. 2000; Tanaka et al. 2001), and the latter action in rat hippocampal CA1 and midbrain dopaminergic neurons is due to the activation of ATP-inhibitable K+ channels following a shortage of ATP (Fujimura et al. 1997; Guatet et al. 1998). Although a change in excitatory transmission following ischemia has been examined in detail in the brain, this has been hardly reported in the spinal cord, to our knowledge.

It is well known that substantia gelatinosa (SG, lamina II of Rexed) neurons of the spinal cord receive glutamatergic transmission from the periphery in a mono- and/or polysynaptic manner, the modulation of which is thought to play an important role in regulating sensory, especially pain transmission (see Willis and Coggeshall 1991 for review). This idea has been supported by the inhibitory actions of opioids (Kohno et al. 1999), serotonin (Ito et al. 2000), and nociceptin (Luo et al. 2002), intrathecal administration of which exhibits antinociception on the excitatory transmission in SG neurons. It is important to address how sensory transmission to SG neurons is affected following ischemia and if so to know whether there is a system to protect SG neurons from the ischemia-induced change in transmission, because this may give a clue to know how to prevent paralytic complications following ischemic stroke. As a first step to this aim, we examined a change in spontaneous excitatory transmission in the SG neurons of adult rat spinal cord slices following superfusion of an oxygen- and glucose-free medium (which simulates ischemia) by use of the conventional whole cell patch-clamp technique. Ischemia in the CNS results in the release of GABA (Sastry Kolluri and Lakshmi 1989; Phillis et al. 1994) as well as L-glutamate.

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METHODS

The method used for obtaining slice preparations of the adult rat spinal cord was similar to that described elsewhere (Ataka et al. 2000; Iyadomi et al. 2000). Briefly, male rats (7–8 wk old) were deeply anesthetized with urethane (1.2 g/kg, ip), and a lumbosacral segment (L1–S3) of the spinal cord was removed and placed in cold (2–4°C) Krebs solution preequilibrated with 95% O<sub>2</sub>–5% CO<sub>2</sub>. The composition of Krebs solution used was (in mM) 117 NaCl, 3.6 KCl, 2.5 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, and 11 glucose. After cutting all ventral and dorsal roots, the pia-arachnoid membrane was removed. The spinal cord was mounted on a Vibratome, and a saturated capillaries (1.5 mm OD), having a resistance of 8–12 MΩ, was completely submerged in the recording chamber and was completely submerged and superfused at a rate of 20–30 ml/min with Krebs solution that was saturated with 95% O<sub>2</sub>–5% CO<sub>2</sub> and maintained at 36.0 ± 0.5°C.

Blind whole cell voltage-clamp recordings were made from SG neurons by using patch-pipettes fabricated from thin walled, fiber-filled capillaries (1.5 mm OD), having a resistance of 8–12 MΩ. The patch-pipette was inserted at the center of SG under visual control, as done previously (Ataka et al. 2000; Iyadomi et al. 2000). The patch-pipette solution used was (in mM) 135 K-gluconate, 0.5 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 5 KCl, 5 EGTA, and 5 HEPES. The holding potential (V<sub>H</sub>) used was −70 mV.

Signals were acquired using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Currents obtained in the voltage-clamp mode were low-pass-filtered at 5 kHz and digitized at 333 kHz with an A/D converter. The data were stored and analyzed with a personal computer using pCLAMP data acquisition program (Version 6.0, Axon Instruments). The program (AxoGraph 4.0, Axon Instruments) used for analyzing spontaneous excitatory postsynaptic currents (sEPSCs) detects spontaneous events if the difference between the baseline and a following current value exceeds a given threshold of 6 pA and separating valleys are <50% of adjacent peaks; a validity of this method was confirmed by measuring visually individual sEPSCs on a fast time scale in all cases. Numerical data are given as the mean ± SE. Statistical significance was determined as P < 0.02 using Student’s t-test (unless otherwise mentioned) or Kolmogorov-Smirnov test. In all cases, n refers to the number of neurons studied.

Ischemia was mimicked by superfusing the slices with a Krebs solution [ischemia-simulating medium (ISM)] equilibrated with 95% N<sub>2</sub>–5% CO<sub>2</sub> where glucose was replaced with an equimolar concentration of sucrose. Drugs were applied by perfusing a solution containing drugs of a known concentration without an alteration in the perfusion rate and temperature; the solution in the recording chamber was normalized in amplitude to that of 59 sEPSCs in the control (continuous line); they are superimposed. V<sub>H</sub> = −70 mV.

RESULTS

Whole cell patch-clamp recordings were made from a total of 93 SG neurons. All SG neurons tested exhibited sEPSCs at

FIG. 1. Effect of superfusing oxygen- and glucose-free (ischemia-simulating) medium (ISM) on glutamatergic spontaneous excitatory postsynaptic currents (sEPSCs) recorded from substantia gelatinosa (SG) neurons. A: ISM-induced changes in holding currents and sEPSCs; the former was composed of an outward current and subsequent slow followed by rapid inward current. In this and following figures, horizontal bars above recordings indicate the period of time during when ISM and/or drugs were superfused. B: 3 consecutive traces of sEPSCs as shown in an expanded scale in time; these were obtained in the control (left), around 2 min (middle), and 4 min (right) after the beginning of ISM superfusion. Each of them was obtained for a period indicated by a bar shown below the recording in A. C: relative frequency of sEPSC following ISM superfusion to that in the control, which is plotted against time. Each point indicates the mean and SE of data obtained for 10 s, which are calculated from 6 different neurons; the SE of the symbol without a vertical bar was within the size of the symbol. D: average of 6 sEPSCs at 4 min after the beginning of ISM superfusion (dotted lines), which was normalized in amplitude to that of 59 sEPSCs in the control (continuous line); they are superimposed. V<sub>H</sub> = −70 mV.
−70 mV where no GABAergic or glycinergic transmission was observed, since the reversal potential for the synaptic currents was near −70 mV, as reported previously (Iyadomi et al. 2000; Lao et al. 2001). Both the amplitude and the frequency of sEPSCs and holding currents were unaffected by TTX (1 μM; n = 4).

Effect of superfusing ISM on holding currents

When superfused with ISM, 57% of 37 SG neurons examined produced an outward current with a latency of 1.1 ± 0.1 min (n = 21); this was followed by a slow and subsequent rapid inward current, as seen in Fig. 1A. This outward current had a peak amplitude of 23 ± 4 pA (n = 21). The remaining neurons (n = 16) had only an inward current (data not shown). Such an inward current (over a holding current in the control) had a time-to-onset of 4.1 ± 0.3 min (n = 37). When ISM was switched to normal Krebs solution in an early phase of the inward current, it was followed by an outward current, and the holding current slowly returned to a level before ISM superfusion, as noted in Fig. 1A. Since such a brief superfusion with ISM had no effect on the amplitude distribution (P = 0.17) while shifting the interval distribution to a shorter one (P < 0.0001; Kolmogorov-Smirnov test). $V_H = −70$ mV.

Effect of superfusing ISM on sEPSCs

When slices were superfused with ISM, sEPSCs were remarkably decreased in a frequency with time, as seen in Fig. 1B. FIG. 2. Effect of bicuculline (Bic; 10 μM) on ISM-induced changes in sEPSCs. A: changes in holding currents and sEPSCs during superfusion of ISM with bicuculline. B: 3 consecutive traces of sEPSCs as shown in an expanded scale in time; these were obtained in the control (left), around 2 min (middle), and 4 min (right) after the beginning of ISM superfusion. Note that bicuculline remarkably increased the frequency of sEPSCs 4 min after the superfusion, compared with the case in the absence of bicuculline (Fig. 1B, right). C: relative frequency of sEPSCs following superfusion of ISM with bicuculline to that in the control, which is plotted against time. Each point indicates data calculated from sEPSCs measured for 10 s. D: cumulative histograms of the amplitude (left) and the inter-event interval (right) of sEPSCs in the control (continuous line) and 4 min (dotted line) in the presence of bicuculline. The histograms were examined during 60 s (222 sEPSC events) in the control and 30 s (552 sEPSC events) around 4 min after ISM superfusion. ISM with bicuculline had no effect on the amplitude distribution ($P = 0.01$) while shifting the interval distribution to a shorter one ($P < 0.0001$; Kolmogorov-Smirnov test). $V_H = −70$ mV.
A and B. Figure 1C demonstrates the time course of a change in the frequency of sEPSCs during ISM superfusion, which is an average of those observed from six cells. It took about 65 s for the frequency to decrease to one-half of that before ISM superfusion. The change in the frequency and the amplitude of sEPSCs was quantitatively analyzed for 10 s in two phases of 2 and 4 min (designated as 1st and 2nd phases, respectively) following the superfusion, in the latter of which the holding current began to shift to the negative side over a level before ISM superfusion. In the first and second phases, the frequency was, respectively, 47 ± 15% and 28 ± 13% (n = 37) of control (12.0 ± 2.0 Hz), while the amplitude was 99 ± 1% and 99 ± 3% of control (15 ± 2 pA; see Fig. 6), respectively. Decay phase of sEPSCs was unchanged by ISM superfusion as seen in Fig. 1D; the half-decay time in the second phase was 104 ± 12% (n = 4) of that (2.9 ± 0.2 ms) in the control.

**Effects of various drugs on the ISM-induced change in sEPSCs**

In the following experiments, ISM was superfused for 4 min, the period of time when a net of ISM-induced inward current began to occur in the absence of drugs.

**GABA ANTAGONISTS.** GABA acts on GABA receptors including ionotropic A-type (GABA\(_A\)) and metabotropic G protein-coupled B-type (GABA\(_B\)) receptors (e.g., see Kumamoto 1997 for review). To know an involvement of the receptors in the ISM-induced decrease in the frequency of sEPSCs, we examined a change in sEPSCs during superfusion of ISM with either GABA\(_A\)-receptor antagonist, bicuculline (10 \(\mu M\)), or GABA\(_B\)-receptor antagonist, CGP35348 (20 \(\mu M\)). As seen in Figs. 2, A and B, and 3, A and B, bicuculline or CGP35348 remarkably increased the fre-
quency from 3.5 to 4 min (2nd phase) but not 2 min (1st phase) after the beginning of ISM superfusion; this increase subsided within about 4 min after its washout. Figures 2C and 3C demonstrate the time courses of their changes in sEPSC frequency in the presence of the antagonists. When assessed quantitatively, in the presence of bicuculline, there was a decrease in the frequency [60 ± 8% (n = 22) of control (11.6 ± 2.1 Hz)] in the first phase without a change in sEPSC amplitude [99 ± 4% (n = 22) of control (22 ± 3 pA)], although this decrement had a tendency to be smaller than that in the absence of the drug. On the other hand, the frequency in the second phase was remarkably increased [by 325 ± 120% (n = 22)]; this was not accompanied by a change in sEPSC amplitude (102 ± 2% of control; see Fig. 6). A similar result was obtained in the presence of CGP35348; there was a decrease and increase in the frequency [49 ± 11% and 426 ± 91% of control (13.0 ± 1.7 Hz, n = 17)] in the first and second phases, respectively, where sEPSC amplitude was not changed [103 ± 3% and 99 ± 2% of control (18 ± 4 pA, n = 17), respectively; see Fig. 6]. Figures 2D and 3D demonstrate cumulative distributions of the amplitude and the inter-event interval of sEPSCs in the second phase during superfusion of ISM with bicuculline and CGP35348, respectively. In each case, a proportion of sEPSCs having a shorter inter-event interval was increased while there was no consistent effect on the cumulative distribution of sEPSC amplitude. Bicuculline (n = 4) or CGP35348 (n = 4) itself did not affect both the amplitude and the frequency of sEPSCs and holding currents in the normoxic condition.

GLYCINE ANTAGONIST. Figure 4, A and B, demonstrates a change in sEPSCs during superfusion of ISM with a glycine-receptor antagonist, strychnine (1 μM). As seen in Fig. 4C, sEPSC frequency was decreased with time; this time course was not different from that in the absence of strychnine (see Fig. 1C). When estimated quantitatively, sEPSC frequencies in the first and second phases were 38 ± 6% and 34 ± 9% of control (12.3 ± 5.7 Hz, n = 5), respectively; the values were not distinct from those without strychnine. There was no change in sEPSC amplitude [101 ± 6% and 99 ± 3% of control (10 ± 1 pA, n = 5), respectively, in the 1st and 2nd phases; see Fig. 6].

TTX. To know an involvement of neuronal activities in the ISM-induced decrease in sEPSC frequency, we examined how TTX (1 μM) affects this change. As seen in Fig. 5,
A–C, TTX remarkably increased the frequency from 3.5 to 4 min (2nd phase) but not first phase during ISM superfusion, an observation similar to that in the presence of bicuculline or CGP35348; this increase subsided within about 4 min after its washout. When estimated quantitatively, sEPSC frequency was decreased to 50 ± 11% (n = 12) of control (10.2 ± 1.1 Hz) in the first phase, while being increased by 328 ± 26% in the second phase. Here, sEPSC amplitude was unchanged in the first and second phases; this was 100 ± 2% and 99 ± 4% of control (12 ± 1 pA; n = 12), respectively. Figure 5D demonstrates cumulative distributions of the amplitude and the inter-event interval of sEPSCs in the second phase during superfusion of ISM with TTX. A proportion of sEPSCs having a shorter inter-event interval was increased, while there was no consistent effect on the cumulative distribution of sEPSC amplitude. Figure 6 summarizes the effects of various drugs on ISM-induced changes in sEPSCs in the second phase.

**DISCUSSION**

This study demonstrated in adult rat SG neurons under simulated ischemia (hypoxia-hypoglycemia) that a slow inward current followed by a rapid inward current (depolarization) was induced with and without a preceding outward current (hyperpolarization); similar currents have been reported in juvenile rat SG neurons (Park et al. 2001) and in rat hippocampal CA1 neurons (Hershkowitz et al. 1993; Tanaka et al. 1997; see Martin et al. 1994 for review). In addition to this change in holding currents, there was a gradual decrease in spontaneous glutamatergic transmission with time. This was presynaptic in origin, because there was...
no change in sEPSC amplitude and in the half-decay time of sEPSCs. Although a rapid increase in sEPSC frequency following hypoxia has been reported in rat hippocampal CA1 neurons (Hershkowitz et al. 1993; Tanaka et al. 2001), this was not noted in SG neurons during superfusion for 4 min with ISM only.

Involvement of GABA receptors and neuronal activities in decrease in spontaneous glutamatergic transmission following ischemia

GABA receptors. When ISM was superfused together with bicuculline or CGP35348, sEPSC frequency reduced by ISM recovered to the control level at 3–3.5 min after the beginning of superfusion. This suggests that GABA_A and GABA_B receptors in nerve terminals of neurons innervating SG neurons may be involved in the ischemia-induced inhibition of the release of L-glutamate from there. In support of this idea, it has been demonstrated that GABAergic interneuron terminals are presynaptic to primary-afferent central terminals (Barber et al. 1978) and interneuron terminals (Magoul et al. 1987) in the rat spinal dorsal horn. The presence of GABA_A receptor subunits in nerve terminals in the rat spinal dorsal horn has been reported by Alvarez et al. (1996). A GABA_B receptor agonist, baclofen, depressed excitatory transmission to SG neurons from primary afferents and interneurons in a presynaptic manner (Ataka et al. 2000; Iyadomi et al. 2000).

Neuronal activities. TTX also produced a recovery similar to those of the GABA antagonists. This suggests that ischemia may induce the release of GABA to glutamatergic nerve terminals in the SG as a result of an increase in the activity of GABAergic interneurons, which in turn leads to a decrease in the release of L-glutamate from there. This idea may be consistent with the observation that TTX depressed ischemia-induced release of GABA from the rat cerebral cortex (Phillis et al. 1994). In support of the idea of GABA release, we have preliminarily observed a remarkable increase in the frequency of spontaneous inhibitory postsynaptic currents following ISM superfusion (see Matsumoto et al. 2001).

Although glycine may exhibit an effect similar to that of GABA, this was not the case, because strychnine did not affect the ischemia-induced decrease in sEPSC frequency. When examined using prisms of the adult rat cortex, ischemia-induced release of L-glutamate was inhibited by GABA through the activation of GABA_A receptors (Nelson et al. 2000). On the other hand, the activation of GABA_B receptors in the rat hippocampal CA1 region did not modify a depression of excitatory transmission following ischemia (Coelho et al. 2000). A role of GABA in protecting CNS neurons from excitotoxicity seems to be distinct among regions in the CNS, probably due to a difference in neuronal architecture such as axo-axonal synapses and in the distribution of GABA_A and GABA_B receptors.

Remarkable increase in glutamatergic transmission following ischemia is unveiled in the presence of GABA antagonists or TTX

It may be noted that the simulated ischemia for 4 min in the presence of either bicuculline, CGP35348, or TTX remarkably increased sEPSC frequency over that in the control. One explanation for this observation is that deprivation of ATP in terminals of glutamatergic neurons innervating SG neurons induces an increase in intraterminal Ca^{2+} concentration as a result of an inhibition in either Ca^{2+} uptake into intracellular stores or Ca^{2+} extrusion to extracellular spaces, leading to an increase in spontaneous release of L-glutamate. Katchman and Hershkowitz (1993) have reported an involvement of intraterminal Ca^{2+} stores in a hypoxia-induced increase in sEPSC frequency. Lack of such an increase in the frequency in SG neurons in the control may have been masked by the above-mentioned action of GABA. Mechanisms for such a remarkable increase in sEPSC frequency in SG neurons remain to be examined.

In conclusion, the present study revealed for the first time in spinal dorsal horn neurons that, following an oxygen- and glucose-free condition, there is an inhibition in the release of L-glutamate, which is in part mediated by the activation of

FIG. 6. Summary of the effects of various drugs on ISM-induced changes in sEPSCs. A and B: relative sEPSC frequency and amplitude at 4 min after ISM superfusion (2nd phase), respectively, to those in the control and in the absence and presence of bicuculline (+Bic; 10 μM; n = 22), CGP35348 (+CGP; 20 μM; n = 17), strychnine (+Str; 1 μM; n = 5), and TTX (+TTX; 1 μM; n = 12). A: *P < 0.02 when compared with that (ISM) in the absence of drugs. B: sEPSC amplitudes in the control were not different from those during ISM superfusion in the absence and presence of drugs.
presynaptic GABA<sub>A</sub> and GABA<sub>B</sub> receptors. It may be noted that in the presence of bicuculline, CGP33548, or TTX, there is still a decrease in sEPSC frequency about 2 min (in the 1st phase) following the in vitro ischemia. It is possible that other neurotransmitters such as adenosine are involved in the remaining decrease of sEPSC frequency, as reported in rat hippocampal CA1 neurons (Tanaka et al., 2001), because adenosine is known to inhibit excitatory transmission to SG neurons (Lao et al., 2001). This remains to be examined.

**Physiological role of GABA in protecting SG neurons from excitotoxicity following ischemia**

Spinal cord injury is a devastating entity in clinical practices including a transient aortic cross-clamp during the operation of thoracoabdominal aneurysm and frequently leads to the secondary damage, resulting in morbidity such as paralytic complications, the main cause of which is ischemia in the spinal cord (Svensson et al., 1993). Such a paralysis may be caused by the ischemia-induced alteration in synaptic transmission, because it has been revealed by using the in vivo patch-clamp technique that SG neurons respond to noxious and innocuous mechanical stimuli given to the periphery (Furue et al., 1999; Narikawa et al., 2000). Endogenous GABA released following ischemia in the spinal cord might serve to protect SG neurons from an excess of L-glutamate and thus to preserve sensory transmission to the SG. Although GABA<sub>A</sub>-receptor agonists appear to provide neuroprotection during ischemia in the brain (see Green et al., 2000; Lyden 1997 for review), this may be so in the spinal dorsal horn.

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**REFERENCES**


