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Actions of Midazolam on GABAergic Transmission in Substantia Gelatinosa Neurons of Adult Rat Spinal Cord Slices

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Background: **Although intrathecal administration of midazolam has been found to produce analgesia, how midazolam exerts this effect is not understood fully at the neuronal level in the spinal cord.**

Methods: **The effects of midazolam on either electrically evoked or spontaneous inhibitory transmission and on a response to exogenous γ-aminobutyric acid (GABA), a $GABA_A$-receptor agonist, muscimol, or glycine were evaluated in substantia gelatinosa neurons of adult rat spinal cord slices by using the whole-cell patch-clamp technique.**

Results: **Bath-applied midazolam (1 μM) prolonged the decay phase of evoked and miniature inhibitory postsynaptic currents (IPSCs), mediated by $GABA_A$ receptors, without a change in amplitudes, while not affecting glycine receptor–mediated miniature inhibitory postsynaptic currents in both the decay phase and the amplitude. Either GABA- or muscimol-induced currents were enhanced in amplitude by midazolam (0.1 μM) in a manner sensitive to a benzodiazepine receptor antagonist, flumazenil (1 μM); glycine currents were, however, unaltered by midazolam.**

Conclusions: **Midazolam augmented both the duration of GABA-mediated synaptic current and the amplitude of GABA-induced current by acting on the $GABA_A$–benzodiazepine receptor in substantia gelatinosa neurons; this would increase the inhibitory GABAergic transmission. This may be a possible mechanism for antinociception by midazolam. (Key words: Benzodiazepine; ion channels; pain.)**

THE dorsal horn of the spinal cord is thought to be an important site for the analgesic action of benzodiazepine receptor agonists. Significant analgesia is produced after intrathecal administration of a water-soluble imidazobenzodiazepine derivative, midazolam, in rats[1-4]; clinical investigations in humans have shown the effectiveness of intrathecally administered midazolam as an analgesic.[5,6] This analgesic effect in rats appears to be mediated through $GABA_A$-benzodiazepine receptors because either a $GABA_A$-receptor antagonist, bicuculline, or a benzodiazepine receptor antagonist, flumazenil, inhibits the midazolam-induced analgesia.[1-3]

Agonists of the $GABA_A$-benzodiazepine receptors are known to modulate synaptic transmission through pre- and postsynaptic mechanisms in neurons of the central nervous system,[7,8] including the spinal dorsal horn.[9] For instance, physiologic evidence shows that benzodiazepines enhance a $GABA_A$ receptor-mediated presynaptic inhibition in central terminals of primary afferent fibers, leading to the decreased release of excitatory neurotransmitters on spinal cord neurons.[10] Alternatively, benzodiazepines potentiate the activation of $GABA_A$ receptors in postsynaptic neurons in the spinal cord, resulting in an inhibition of excitatory transmission. Such pre- and postsynaptic actions of benzodiazepines are supported by binding and histochemical studies. The highest density of $GABA_A$-benzodiazepine receptors has been found in superficial layers of the spinal cord, especially in the lamina II of Rexed[11] (substantia gelatinosa [SG]), both in

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rats[12,13] and in humans.[14,15] After a neonatal capsaicin treatment that is known to destroy fine primary afferent fibers, the binding sites of a $GABA_A$-receptor agonist, muscimol, in the superficial dorsal horn were reduced in number but not abolished,[16] indicating the presence of $GABA_A$ receptors in both central terminals of primary afferent fibers and interneurons in the SG.[13] Alternatively, it has been shown that SG neurons contain abundant endogenous GABA that may play a role in the control of pain as a major inhibitory neurotransmitter.[17]

Substantia gelatinosa neurons preferentially receive thin myelinated Aδ and unmyelinated C primary afferent fibers, both of which carry nociceptive information and, therefore, are thought to play an important role in modulating nociceptive transmission.[18,19] It is possible that the midazolam-induced inhibition of pain transmission is caused by a modulation of inhibitory transmission in the SG. The transmission is thought to occur through the activation of GABA- and glycine-containing interneurons that receive Aδ-fiber inputs from the periphery.[20] The aim of the current study was to evaluate the actions of midazolam on the inhibitory transmission and also on the currents produced by the application of GABA, muscimol, or glycine by using blind patch-clamp recordings from SG neurons of adult rat spinal cord slices.

Materials and Methods

Preparation of Spinal Cord Slice

This study was approved by the institutional Animal Research Committee of the Niigata University School of Medicine. The methods used for obtaining an adult rat spinal cord slice and for blind patch-clamp recordings from SG neurons have been described elsewhere.[21–24] Briefly, male adult Sprague-Dawley rats (6–8 weeks old) were anesthetized with urethane (1.5 g/kg, intraperitoneal), and then lumbosacral laminectomy was performed. The lumbosacral spinal cord (L1–S3) was removed and placed in preoxygenated Krebs solution at 1–3°C. After cutting all of ventral and dorsal roots, the pia-arachnoid membrane was removed. The spinal cord was mounted on a vibrating microslicer (DTK1500; Dosaka Co. Ltd., Kyoto, Japan) and then a 500-μm thick transverse slice was cut. The slice was placed on nylon mesh in the recording chamber, which had a volume of 0.5 ml, then perfused at a rate of 15 ml/min with Krebs solution saturated with 95% O_2 and 5% CO_2, and maintained at 36 ± 1°C; this perfusion was continued for at least 1 h before recording. The Krebs solution contained 117 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose.

Recording and Stimulation

Substantia gelatinosa neurons were identified by location and morphologic features, as reported previously.[19,22,25] Under a binocular microscope and with transmitted illumination, the SG was clearly discernible as a relatively translucent band across the dorsal horn. Blind whole-cell patch-clamp recordings were made from the SG neurons with patch-pipette electrodes having a resistance of 5–10 MΩ.[21–24] The patch-pipette solution contained 110 mM cesium sulfate, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM EGTA, 5 mM HEPES, 5 mM tetraethylammonium (TEA), 5 mM adenosine triphosphate-Mg salt, and 1 mM guanosine 5′-O-(2-thiodiphosphate) (GDP-β-S), where GDP-β-S and potassium (K^+) channel blockers (cesium and tetraethylammonium) were added to inhibit the activation of a $GABA_B$ receptor through the action of guanosine triphosphate-binding proteins in postsynaptic neurons and to block activation of K^+ channels that may result from the postsynaptic effect, respectively. Signals were gained using an Axopatch 200A 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 using an A/D converter. Data were stored and analyzed using a personal computer using pCLAMP6 and Axo-Graph data acquisition programs (Axon Instruments).

Monosynaptic inhibitory postsynaptic currents (IPSCs) were evoked in the presence of a non-*N*-methyl-D-aspartate (NMDA)-receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and an NMDA-receptor antagonist, DL-2-amino-5-phosphonovaleric acid (APV), at a frequency of 0.1 Hz by focal stimulation of interneurons in the SG with a monopolar silver-wire electrode (50 μm diameter), insulated except for the tip, located within 150 μm from the recorded neurons.

Application of Drugs

Drugs were applied by superfusion, with a change in solutions in the recording chamber being completed within 15 s. Drugs used were GABA, muscimol, glycine, APV, strychnine, bicuculline, GDP-β-S (Sigma, St. Louis, MO), CNQX (Tocris Cookson, St. Louis, MO), midazolam, flumazenil (given from Hoffmann-LaRoche, Basel, Switzerland), and tetrodotoxin (TTX; Wako, Osaka, Ja-

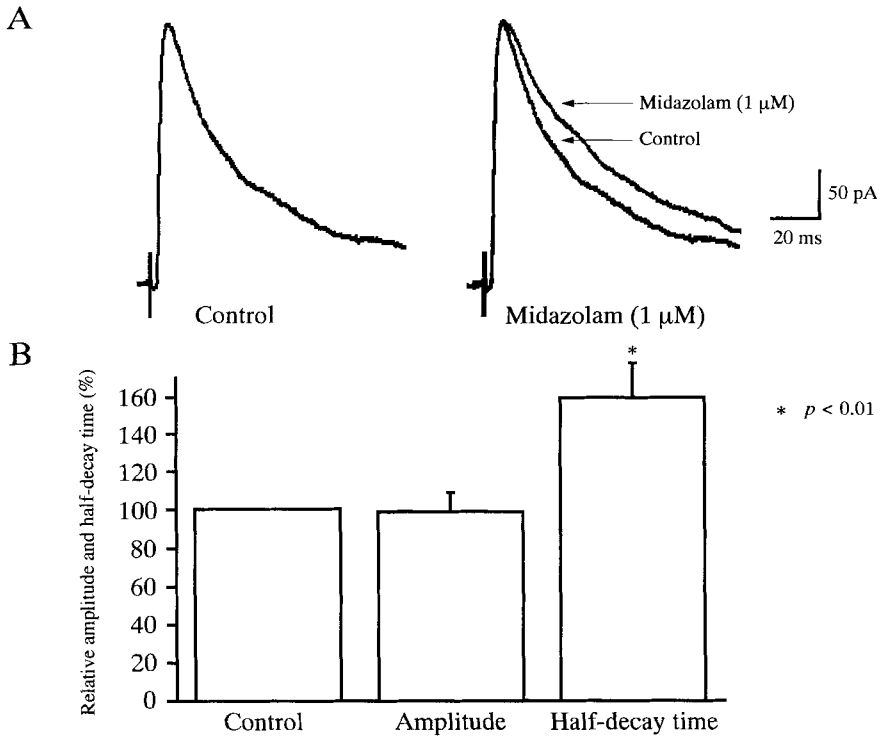

Fig. 1. Effect of midazolam (1 μM) on GABAergic evoked inhibitory postsynaptic currents (eIPSCs) in substantia gelatinosa (SG) neurons. (*A*) Averaged traces of six consecutive GABAergic eIPSCs before (*left*; control) and 2 min during (*right*; the control eIPSC is superimposed for comparison) the application of midazolam. (*B*) Amplitude and half-decay time of GABAergic eIPSCs under the action of midazolam, relative to those in the control (n = 8). Vertical bars show SD; *Significantly different from control, $P < 0.01$. The eIPSCs were evoked every 10 s in the presence of strychnine (2 μM) together with CNQX (20 μM) and APV (50 μM). Holding potential (V_H) = 0 mV.

pan). The application of GABA was performed at least 10 min after the establishment of whole-cell configuration when a $GABA_B$ response was not observed.[26]

Statistical Analysis

All numerical data were given as the mean ± SD. Statistical significance was determined as $P < 0.01$ using either the paired Student *t* test (unless otherwise noted) or the Kolmogorov-Smirnov test. In all cases, n refers to the number of neurons studied.

Results

Whole-cell recordings were obtained from 72 SG neurons. Stable recordings could be obtained from slices maintained *in vitro* for more than 10 h, and recordings were made from a single SG neuron for up to 4 h. All SG neurons evaluated exhibited spontaneous excitatory postsynaptic currents (EPSCs) and IPSCs at holding potentials (V_Hs) of −70 mV and more positive (depolarized) than −60 mV, respectively.[21,23,24]

After a blockade of glutamatergic transmission by adding CNQX (20 μM) and APV (50 μM) to the Krebs solution, a local stimulation of interneurons in the SG-induced monosynaptic IPSCs in SG neurons. The evoked IPSCs (eIPSCs) consisted of two components that are distinct in duration and pharmacology: a long eIPSC, which had a half-decay time of 29.6 ± 8.4 ms and was depressed by bicuculline (20 μM); and a short eIPSC, which had a half-decay time of 6.9 ± 3.6 ms and was abolished by a glycine receptor antagonist, strychnine (2 μM), indicating that they are mediated by $GABA_A$ and glycine receptors, respectively (figs. 1A and 5A).[20] The GABAergic and glycinergic eIPSCs had mean amplitudes of 153 ± 49 pA (range, 99-270 pA; n = 22) and 211 ± 67 pA (range, 96-350 pA; n = 16; V_H = 0 mV), respectively.

Miniature IPSCs (mIPSCs) were isolated by adding tetrodotoxin (0.5 μM), together with CNQX (20 μM) and APV (50 μM), to the superfusing solution. There were two distinct types of mIPSCs at the V_H of 0 mV. GABAergic mIPSCs had a relatively long duration (50-100 ms) and were antagonized by bicuculline (20 μM), and glycinergic mIPSCs had a short duration (20-40 ms) and were antagonized by strychnine (2 μM), indicating that they are mediated by $GABA_A$ and glycine receptors, respectively,[21,23,24] as seen for eIPSCs.

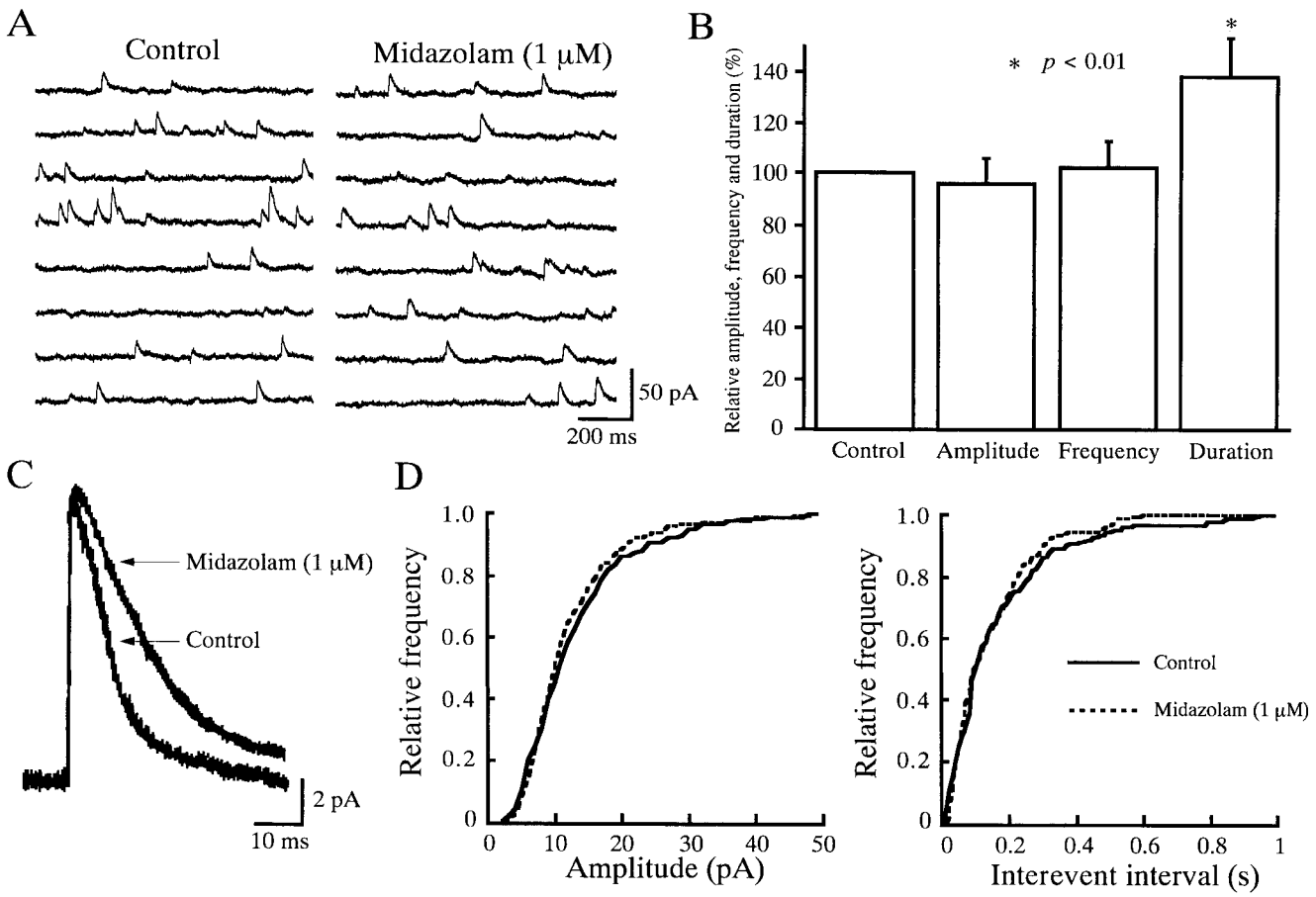

Fig. 2. Effect of midazolam (1 μM) on GABAergic miniature inhibitory postsynaptic currents (mIPSCs) in substantia gelatinosa (SG) neurons. (*A*) Eight consecutive traces of GABAergic mIPSCs before (*left*) and 2 min during (*right*) the application of midazolam. (*B*) Amplitude, frequency, and duration of GABAergic mIPSCs measured for 1 min under the action of midazolam, relative to those in the control (n = 7). Vertical bars show SD; *Significantly different from control, $P < 0.01$. (*C*) Averaged traces of 174 and 200 GABAergic mIPSCs before and after the addition of midazolam, respectively; these are superimposed for comparison. (*D*) Cumulative distributions of the amplitude (*left*) and interevent interval (*right*) of GABAergic mIPSCs, before (continuous line) and under (dotted line) the action of midazolam, which were obtained by analyzing 174 and 200 mIPSC events (which occurred for 30 s), respectively. Midazolam had no effect on both distributions of the amplitude and frequency ($P > 0.4$, Kolmogorov-Smirnov test). The mIPSCs were recorded in the presence of strychnine (2 μM), together with tetrodotoxin (0.5 μM), CNQX (20 μM), and APV (50 μM). $V_H = 0$ mV.

Effect of Midazolam on GABAergic Transmission in Substantia Gelatinosa Neurons

Superfusing midazolam (0.1 μM) did not affect the peak amplitude (99 ± 8%, n = 14, of control; $P > 0.05$; not shown) of GABAergic eIPSC recorded in the presence of strychnine (2 μM). At a higher concentration of 1 μM, midazolam still had no effect on the amplitude (99 ± 9%, n = 8, of control; $P > 0.05$). The half-decay time of eIPSC was, however, markedly increased by midazolam (1 μM) to 159 ± 17% of control ($P < 0.01$), as seen in figure 1.

The average frequency of GABAergic mIPSCs was 5.1 ± 1.4 Hz (range: 4.1–7.4 Hz) and amplitude was 12.6 ± 4.6 pA (range, 9.3–20.5 pA; n = 7) at 0 mV. Figure 2 shows an action of midazolam (1 μM) on the mIPSC. Midazolam had no effect on frequency and amplitude of mIPSC (fig. 2B) and also on the cumulative distributions (fig. 2D). The mIPSC was, however, enhanced in duration by midazolam to 138 ± 14% of control (n = 7; $P < 0.01$; figs. 2B and 2C), as seen for GABAergic eIPSCs. In the case of the mIPSC, we measured duration instead of half-decay time, because this amplitude was very small and hence it was difficult to measure the half-decay time.

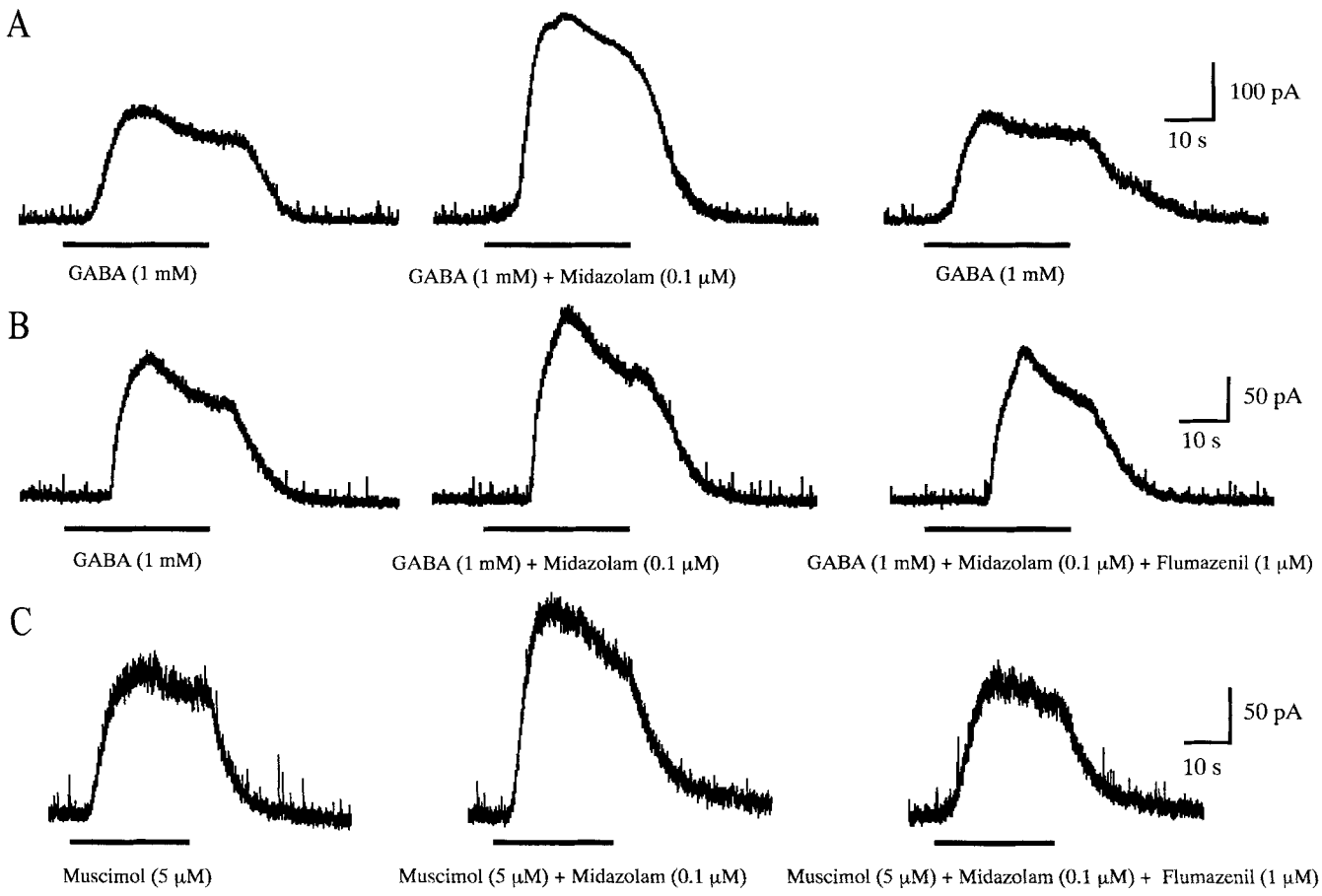

Fig. 3. Potentiation by midazolam (0.1 μM) of a current evoked in substantia gelatinosa (SG) neurons by bath application of either GABA (1 mM) or muscimol (5 μM) and antagonism of this action by flumazenil (1 μM). (*A*) GABA currents before (*left*) the action of midazolam, during (*middle*) the action, and after washout (*right*). (*B*) GABA current (*left*) was enhanced by midazolam (*middle*); this action was not observed in the presence of flumazenil (*right*). (*C*) Muscimol current (*left*) was enhanced by midazolam (*middle*); this action was not observed in the presence of flumazenil (*right*). In this and in the following figures, the horizontal bars below the records indicate the period of time during which the drugs were applied. V_H = 0 mV.

Effect of Midazolam on either GABA- or Muscimol-induced Current in Substantia Gelatinosa Neurons

Superfusing either GABA (1 mM) or muscimol (5 μM) elicited an outward current at 0 mV in SG neurons. When applied together with midazolam (0.1 μM), the GABA or muscimol current was markedly potentiated in peak amplitude, as shown in figure 3. The augmentative magnitudes averaged 52 ± 18% (n = 8) and 36 ± 14% (n = 9), respectively ($P < 0.01$; fig. 4). These effects were blocked by a benzodiazepine receptor antagonist, flumazenil (1 μM; figs. 3B and 3C), indicating an activation of $GABA_A$-benzodiazepine receptors. Superfusing midazolam at concentrations of 0.1–1 μM alone did not produce any currents in SG neurons.

Lack of the Effect of Midazolam on Glycinergic Transmission and Glycine-induced Current in Substantia Gelatinosa Neurons

Superfusing midazolam at concentrations of 0.1–1 μM did not affect either the peak amplitude or the half-decay time of glycinergic eIPSC recorded in the presence of bicuculline (20 μM) (figs. 5A and 5B). A glycine-induced current was also unchanged by midazolam (0.1 μM; figs. 5C and 5D).

Discussion

The current study revealed that midazolam had no effect on the amplitude of GABAergic eIPSC and mIPSC

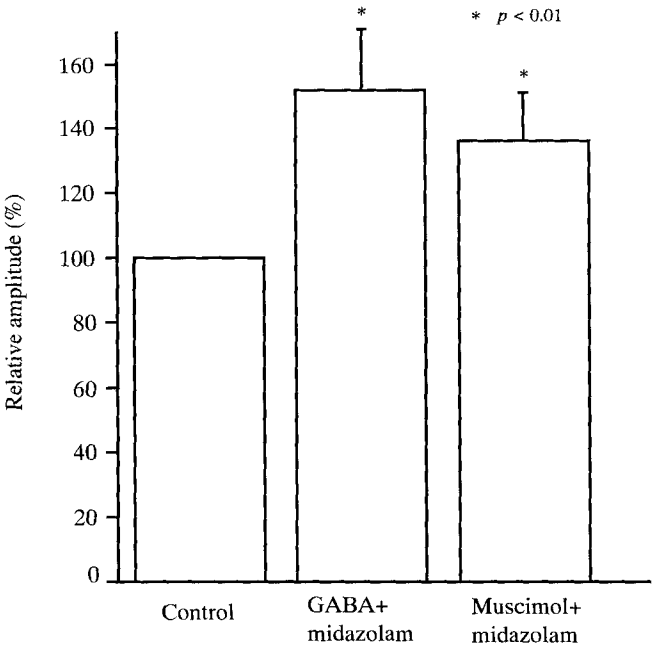

Fig. 4. Amplitude of either GABA- (1 mM) or muscimol-induced (5 μM) current (n = 8 and 9, respectively) under the action of midazolam (0.1 μM) in substantia gelatinosa (SG) neurons, relative to that in the control. Vertical bars show SD; *Significantly different from control, $P < 0.01$.

or on the frequency of the mIPSC, but prolonged duration; glycinergic transmission was never affected in amplitude and duration in SG neurons of the adult rat spinal dorsal horn. A similar effect of benzodiazepines on GABAergic mIPSC has been observed in rat hippocampal neurons in slices.[27,28] Conversely, midazolam enhanced the amplitude of either GABA or muscimol current, as shown in rodent hippocampal neurons.[29,30] This action was antagonized by the benzodiazepine receptor antagonist, flumazenil, indicating the activation of the $GABA_A$-benzodiazepine receptor.

Difference in the Actions of Midazolam between GABAergic IPSC and GABA Current in Substantia Gelatinosa Neurons

There are several possibilities for the difference in the action of midazolam between exogenous and synaptic GABA responses. First, midazolam may positively modulate extrasynaptic but not synaptic $GABA_A$-benzodiazepine receptors in SG neurons. There is a variety of $GABA_A$-receptor subunits, such as α_1-α_6, β_1-β_4, γ_1-γ_4, δ, and ϵ, a different combination of which results in forming $GABA_A$ receptors that exhibit a distinct pharmacologic property, including benzodiazepine actions.[7,31-33] Naturally expressed $GABA_A$ receptors are thought to be heteromeric and comprise forms of the α, β, and γ subunits; a $GABA_A$-receptor current is not potentiated by benzodiazepines unless the γ_2 subunit is added to the α and β subunits, although the nature of the α subunits affects the benzodiazepine sensitivity.[8,16,34] Immunohistochemical studies have shown that a $GABA_A$-receptor heterogeneity occurs within the laminae of the spinal cord.[12] In support of an idea that there may be a different type of receptor activated by GABA between the synaptic and extrasynaptic sites of SG neurons, Park *et al.*[26] have reported a GABA response caused by the activation of chloride (Cl^-) channels that is resistant to bicuculline (100 μM), as seen for the $GABA_C$ receptor,[33,35] while being augmented by a benzodiazepine, flunitrazepam. Such a bicuculline-insensitive GABA response was never synaptically elicited.[21] Second, augmentation of IPSC by midazolam may have been occluded because of the release of GABA into the synaptic cleft at a concentration high enough to saturate $GABA_A$ receptors in postsynaptic neurons, because a single quantum of GABA is known to saturate postsynaptic $GABA_A$ receptors.[28] Such an occlusion appears not to occur for the activation of extrasynaptic $GABA_A$ receptors by the application of GABA at a high concentration, such as 1 mM, because the responses of SG neurons to GABA are enhanced in amplitude with an increase in GABA concentrations up to 10 mM.[26]

Prolongation by Midazolam of GABAergic IPSC in Substantia Gelatinosa Neurons

This is unlikely because of an inhibition of GABA uptake by midazolam,[36] because muscimol and GABA current are enhanced in amplitude by midazolam. This prolongation would be caused by an increase either in the frequency of $GABA_A$-receptor channel opening or in the single-channel conductance, as shown in other types of neurons.[7,37] A prolongation of the time course of GABAergic IPSC could reduce the likelihood of generating an action potential in postsynaptic SG neurons, resulting in less excitation of projection neurons and, thus, in antinociception.

Lack of the Effects of Midazolam on Glycinergic Transmission in Substantia Gelatinosa Neurons

In addition to GABA, glycine is a major inhibitory neurotransmitter of importance in the mammalian

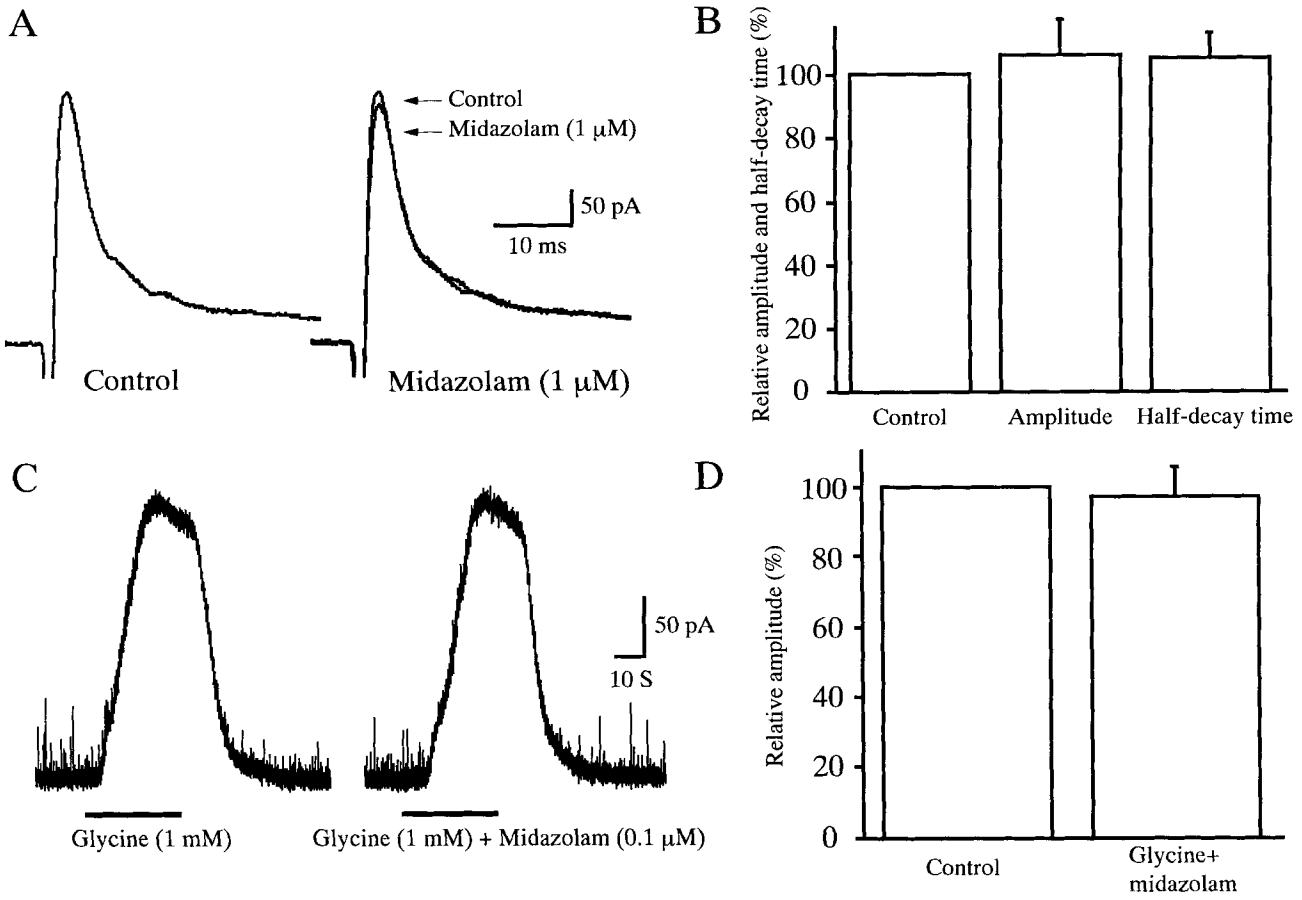

Fig. 5. Effect of midazolam (0.1–1 μM) on either glycinergic evoked inhibitory postsynaptic currents (eIPSCs) or glycine-induced (1 mM) currents in substantia gelatinosa (SG) neurons. (*A*) Averaged traces of six consecutive glycinergic eIPSCs before (*left*; control) and 2 min during (*right*; where the control eIPSC is superimposed for comparison) the application of midazolam (1 μM). (*B*) Amplitude and half-decay time of glycinergic eIPSCs under the action of midazolam (1 μM), relative to those in the control (n = 6; insignificant with $P > 0.05$). A similar result was obtained by midazolam (0.1 μM; not shown). The eIPSCs were evoked every 10 s in the presence of bicuculline (20 μM), together with CNQX (20 μM) and APV (50 μM). (*C*) Glycine currents before (*left*) and during (*right*) the action of midazolam (0.1 μM). (*D*) Amplitude of glycine current under the action of midazolam (0.1 μM), relative to that in the control (n = 10; insignificant with $P > 0.05$). Vertical bars in *B* and *D* show SD. V_H = 0 mV.

central nervous system. The glycine and the $GABA_A$ receptor are coupled to a Cl^- channel. The SG has been reported to contain glycine-like immunoreactive neurons[17] and also glycine-receptor-like immunoreactive neurons.[38] Considering a homology in amino acid sequence between these receptors, some drugs, including benzodiazepines, may affect both in a similar fashion in the SG.[36] Our current results, however, showed that the glycinergic transmission is not affected by midazolam. It is suggested that the glycine receptor located in SG neurons, which may inhibit nociceptive transmission, is not a primary target for midazolam.

Physiologic Significance of the Midazolam Actions on Substantia Gelatinosa Neurons

The dorsal horn of the spinal cord, especially SG, is a primary receiving area for somatosensory (presumably nociceptive) inputs that contains a high concentration of $GABA_A$ receptors and also of endogenous GABA.[13] GABA and L-glutamate may be involved in nociceptive transmission in SG neurons. Applying $GABA_A$-receptor antagonists often leads to a bursting activity of excitatory postsynaptic potentials in SG neurons in response to a single stimulus that previously evoked only a single excitatory postsynaptic potential, suggesting that a normally inhibitory circuitry in the SG may prevent a recur-

rent excitation.[20,39] A low dose of intrathecally applied midazolam produces clinically effective and localized analgesia[5,6]; this would be caused by an action of midazolam in the spinal dorsal horn. Midazolam may increase GABAergic transmission there, as shown in the current study. A tonic inhibition of GABA, mediated by extrasynaptic $GABA_A$ receptors, also may be enhanced by midazolam, because this drug potentiates a muscimol and a GABA response. It is unknown whether the bicuculline-resistant GABA response at the extrasynaptic site[26] is modulated by midazolam. In molecular analyses, an endogenous ligand for the benzodiazepine receptor has been proposed.[40] The current study provides a further possible physiologic underpinning for behavioral studies that have shown the antinociceptive effect of spinal benzodiazepine agonists.[1-4]

In summary, the results presented herein provide a cellular basis for the antinociceptive action of midazolam at the spinal cord level and support a physiologic role of midazolam as important inhibitory drugs of sensory information processing in the spinal dorsal horn.

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