Inhibition of Glutamatergic Synaptic Input to Spinal Lamina IIo Neurons by Presynaptic $\alpha_2$-Adrenergic Receptors

YU-ZHEN PAN,1 DE-PEI LI,1 AND HUI-LIN PAN1,2

1Department of Anesthesiology and 2Department of Neuroscience and Anatomy, Penn State University College of Medicine, Hershey, Pennsylvania 17033-0850

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INTRODUCTION

The dorsal horn of the spinal cord is an important site for the relay of nociceptive information. Activation of the descending noradrenergic system can inhibit the transmission of nociceptive information through $\alpha_2$-adrenergic receptors located in the spinal dorsal horn. For instance, the analgesic effect produced by stimulation of the descending noradrenergic system is blocked by intrathecal injection of $\alpha_2$-receptor antagonists (Budai et al. 1998; Nuseir and Proudfoot 2000; Yaksh 1985). Furthermore, intrathecal administration of clonidine, an $\alpha_2$-adrenergic receptor agonist, produces antinociceptive effects in acute and chronic pain models (Buerkle and Yaksh 1998; Pan et al. 1999; Yaksh et al. 1995). Intrathecal and epidural administration of clonidine has been used clinically for pain relief (De Kock et al. 1997; Rauck et al. 1993). However, the precise site and mechanisms underlying the potent analgesic action of spinally administered $\alpha_2$-adrenergic receptor agonists are not fully known.

The $\alpha_2$-adrenergic receptors are located in the superficial dorsal horn, and the $\alpha_{2A}$ receptor subtype is predominantly located on the terminals of primary C-fiber afferents (Roulet et al. 1994; Stone et al. 1998; Sullivan et al. 1987). The dorsal horn neurons receive both excitatory and inhibitory synaptic inputs from primary afferent nerves, interneurons, and nerve terminals projected from neurons in supraspinal nuclei (De Biasi and Rustioni 1988; Headley and Grillner 1990; Lekan and Carlton 1995; Tachibana et al. 1994). The excitatory amino acid, glutamate, is a major neurotransmitter of nociception from peripheral nociceptors to the dorsal horn neurons (Dougherty and Willis 1991, 1992; Stanfa and Dickinson 1999). It has been proposed that inhibition of the nociceptive input from primary afferents to dorsal horn neurons contributes to the analgesic actions produced by activation of the descending noradrenergic system and $\alpha_2$-adrenergic receptor agonists. In this regard, clonidine significantly reduces glutamate release from spinal synaptosomes and slices evoked by KCl and capsaicin (Kamisaki et al. 1993; Ueda et al. 1995), suggesting that activation of $\alpha_2$-adrenergic receptors inhibits the excitatory synaptic transmission in the spinal cord. However, the importance of presynaptic $\alpha_2$-adrenergic receptors in the regulation of the glutamatergic synaptic input to dorsal horn neurons has not been demonstrated directly. Baba et al. (2000) recently reported that norepinephrine has no effect on the excitatory glutamatergic input to the dorsal horn neurons in the lamina II (substantia gelatinosa). Thus the electrophysiological data seem to be contrary to those obtained from the neurochemistry studies (Kamisaki et al. 1993; Ueda et al. 1995). The reasons underlying this discrepancy are not entirely clear. A recent neuroanatomical study provides strong evidence challenging the monolithic treatment of the spinal lamina II neurons in...
previous spinal slice recording studies. Woodbury et al. (2000) have found that the spinal lamina II of mammals subserves a clear duality of function, with only the outer zone of the lamina II (lamina IIo) receiving the C-fiber afferent input. This suggests that lamina IIo neurons may have different functions than those in the inner zone of lamina II (lamina IIi) in mediation of nociception. Because spinal α2A- and α2C-adrenergic receptors are located primarily on the capsaicin-sensitive C-fiber afferents (Stone et al. 1998), it is possible that the spinal α2-adrenergic receptors may only affect the glutamatergic input to lamina IIo neurons. Consequently, it is important to further determine the effect of α2-adrenergic receptor agonists on glutamatergic synaptic input to lamina IIo neurons. In the present study, by directly recording postsynaptic currents from lamina IIo neurons in the rat spinal cord slice, we tested a hypothesis that activation of presynaptic α2-adrenergic receptors by clonidine inhibits the glutamatergic synaptic input to lamina IIo neurons.

METHODS

Spinal cord slice preparation

Sprague-Dawley rats (4–6 wk old, Harlan Industries, Indianapolis, IN) were used for this study. The lumbosacral segment of the spinal cord was rapidly removed through a limited laminectomy under halothane anesthesia and placed in a preoxygenated ice-cold sucrose artificial cerebrospinal fluid (ACSF). The sucrose ACSF was composed as follows (in mM): 234 sucrose, 3.6 KCl, 1.2 MgCl2, 2.5 CaCl2, 1.2 NaH2PO4, 12.0 glucose, and 25.0 NaHCO3. After the dura mater was completely removed, the lumbosacral segment of the spinal cord was placed in a shallow groove formed in a gelatin block and then glued on the stage of a vibratome (Technical Products International, St. Louis, MO). The resistance of the pipette tip was 5–10 MΩ when filled with the intracellular solution containing (in mM): 135.0 potassium gluconate, 5 KCl, 2.0 MgCl2, 0.5 CaCl2, 5.0 HEPES, 5.0 EGTA, 5.0 ATP-Mg, and 0.5 Na-GTP; adjusted to pH 7.2–7.4 with 1 M of KOH (290–300 mOsm). The slice was placed in a glass-bottomed chamber (Warner Instruments, Hamden, CT) and fixed with a grid of parallel nylon threads supported by a U-shaped stainless steel weight. The slice was perfused at 5.0 ml/min at 36°C maintained by an in-line solution heater and a temperature controller (TC-324, Warner Instruments).

Positive pressure was continuously applied to the recording pipette, which was advanced toward the identified neuron through a motorized manipulator (MP285, Sutter Instrument) under direct visual control. Once the pipette touched the membrane of the neuron, the pressure was immediately released, and slight negative pressure was applied to establish a high-resistance seal. The cell membrane was then ruptured by further suction to record in the whole cell configuration. Recordings of postsynaptic currents began 5 min later after the whole cell access was established and the current reached a steady state. The excitatory postsynaptic currents (EPSCs) were recorded using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) at a holding potential of −70 mV (Li and Pan 2001). Signals were filtered at 1–2 kHz, digitized at 10 kHz (DigiData 1320A, Axon Instruments), and recorded into a Pentium computer using the pCLAMP 8.01 program. Membrane potentials were not corrected for liquid junction potentials between the Krebs and patch-pipette solutions. All miniature excitatory postsynaptic currents (mEPSCs) were recorded in the

Recordings of excitatory postsynaptic currents

Recordings of postsynaptic currents were performed in an RF-shielded room using the whole cell voltage-clamp method, similar to what we described previously (Li and Pan 2001). The lamina II has a distinct translucent appearance and can be easily distinguished under the microscope (Woodbury et al. 2000; Yoshimura and Nishi 1993). Based on their location described previously (Woodbury et al. 2000), the lamina II neurons in the spinal cord slice were visualized and identified (Fig. 1) under a fixed-stage microscope (BX50WI, Olympus, Japan) with the differential interference contrast/infrared illumination. The image of neurons in the lamina II was captured and enhanced through a CCD camera and displayed on a video monitor. We restricted our recordings to neurons located in the lamina IIo. The electrode for the whole cell recordings was triple-pulled with a puller (P-97, Sutter Instrument, Novato, CA) using borosilicate glass capillaries (OD 1.2 mm; ID 0.86 mm; World Precision Instruments, Sarasota, FL). The resistance of the pipette tip was 5–10 MΩ when filled with the intracellular solution containing (in mM): 135.0 potassium gluconate, 5 KCl, 2.0 MgCl2, 0.5 CaCl2, 5.0 HEPES, 5.0 EGTA, 5.0 ATP-Mg, and 0.5 Na-GTP; adjusted to pH 7.2–7.4 with 1 M of KOH (290–300 mOsm). The slice was placed in a glass-bottomed chamber (Warner Instruments, Hamden, CT) and fixed with a grid of parallel nylon threads supported by a U-shaped stainless steel weight. The slice was perfused at 5.0 ml/min at 36°C maintained by an in-line solution heater and a temperature controller (TC-324, Warner Instruments).

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FIG. 1. A: a brightfield photomicrograph (×100) showing the location of lamina II in the dorsal horn of a lumbar spinal cord slice. Note that the lamina II has a distinct translucent appearance under the microscope. DR, attached dorsal root. B: a photomicrograph (×600) showing a recorded outer zone of lamina II (lamina IIo) neuron (*) and the pipette electrode (×) viewed under the microscope using differential interference contrast and infrared illumination.

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presence of tetrodotoxin (TTX, 1 μM), bicuculline (10 μM), and strychnine (5 μM).

The evoked EPSCs (eEPSCs) from lamina IIo neurons were induced by electrical stimulation (0.3–0.5 ms, 0.2 mA, and 0.2 Hz) of the dorsal root entry zone or the attached dorsal root through a bipolar tungsten electrode connected to a stimulator (World Precision Instruments, Sarasota, FL). Recordings of eEPSCs were similar to mEPSCs as described in the preceding text except that TTX was not used (Yoshimura and Nishi 1993). In some experiments, miniature inhibitory postsynaptic currents (mIPSCs) of lamina IIo neurons were recorded in the presence of TTX and CNQX at a holding potential of −70 mV. The internal pipette solution for the mIPSC recording contained (in mM): 140.0 KCl, 1.0 MgCl2, 1.0 CaCl2, 10.0 HEPES, 10.0 EGTA, 5.0 ATP-Mg, and 0.5 Na-GTP; adjusted to pH 7.2–7.4 with 1 M of KOH (290–300 mOsm).

**Experimental protocols**

The resting membrane potential and the input resistance were continuously monitored throughout the recording period. Recordings were abandoned if the input resistance changed >15% (Li and Pan 2001). After recording the mEPSCs of lamina IIo neurons for 5 min as the baseline control, 10 μM (final concentration) of clonidine was perfused into the slice for 3–5 min. Then the mEPSCs were recorded for 5 min during clonidine perfusion. In separate lamina IIo neurons, we determined the role of α2C- and α1-adrenergic receptors in the effect of clonidine on mEPSCs of lamina IIo neurons. Two micromolar of yohimbine, an α2-adrenergic receptor antagonist (Miyazaki et al. 1998; Ueda et al. 1995), or 2 μM of prazosin, an α1-adrenergic receptor antagonist (Miyazaki et al. 1998; Yakh 1985; Yakh et al. 1995), was first applied to the slice chamber for 3 min followed by perfusion of 10 μM of clonidine plus yohimbine or prazosin. Also, to examine the potential effect of clonidine on the inhibitory synaptic input to lamina IIo neurons, the effect of 10 μM of clonidine on mIPSCs was measured using a similar protocol as described in the preceding text.

To further assess the effect of clonidine on glutamate released from the central terminals of primary afferent nerves, the eEPSCs were recorded from additional lamina IIo neurons. The amplitude of eEPSCs was measured during control and applications of 1–20 μM of clonidine. The effects of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM) or yohimbine (2 μM) plus clonidine (10 μM) on eEPSCs were also tested in some lamina IIo neurons. TTX, CNQX, bicuculline methiodide, strychnine, clonidine, and yohimbine were obtained from Sigma (St. Louis, MO). Prazosin was purchased from Tocris Cookson (Ballwin, MO). Drugs were dissolved in the Krebs solution and perfused into the slice chamber using the syringe pumps (Razel Scientific Instruments, Stamford, CT).

**Data analysis**

Data are presented as means ± SE. The mEPSCs and mIPSCs were analyzed off-line with a peak detection program (MiniAnalysis, Synaptosoft, Leonia, NJ). The cumulative probability of the amplitude and inter-event interval were compared by the Komogorov-Smirnov test, which estimates the probability that two cumulative distributions are similar (Sulzer and Pothos 2000). Analyses of the effects of drugs on the amplitude of eEPSCs were performed using Clampfit (Axon Instruments). The effects of drugs on the amplitude and frequency of mEPSCs and mIPSCs were determined by Wilcoxon signed-rank test or nonparametric ANOVA (Kruskal-Wallis or Friedman) test with Dunn’s post hoc test. P < 0.05 was considered to be statistically significant.

**Results**

Spontaneous mEPSCs were recorded from a total of 57 lamina IIo neurons. Stable recordings could be obtained from slices maintained in vitro for >6 h. Once the whole cell recording was established, the mEPSCs often could be recorded for ≥30 min without noticeable changes in the resting membrane potential and input resistance. In the presence of TTX (1 μM), bicuculline (10 μM), and strychnine (5 μM), the amplitude of mEPSCs ranged from 9.6 to 39.4 pA (19.7 ± 0.9 pA) and the frequency of mEPSCs varied from 0.3 to 18.7 Hz (5.2 ± 0.6 Hz, n = 57). The mEPSCs from a total of 13 lamina IIo neurons were studied. In the presence of bicuculline and strychnine, the peak amplitude of eEPSCs ranged from 82.1 to 504.3 pA (257.7 ± 38.1 pA, n = 13).

**Effect of clonidine on mEPSCs and mIPSCs**

Application of 10 μM of clonidine significantly decreased the frequency of mEPSCs of 27 lamina IIo neurons from 5.8 ± 0.9 to 2.7 ± 0.6 Hz (P < 0.05, Fig. 2). However, clonidine did not significantly alter the amplitude (21.8 ± 1.6 vs. 21.2 ± 1.5 pA) and the decay time constant (1.8 ± 0.1 vs. 1.8 ± 0.1 ms, Fig. 2) of mEPSCs in 25 of 27 lamina IIo neurons. Application of 20 μM of CNQX abolished mEPSCs of all lamina IIo neurons tested (Fig. 2A). The cumulative probability analysis of mEPSCs revealed that the distribution pattern of the inter-event interval shifted toward right in response to clonidine (Fig. 2C), but the distribution pattern of the amplitude was not affected by clonidine (Fig. 2B). The effect of clonidine on mEPSCs was further analyzed by measuring the time constant of the decay phase of the mEPSCs. The decay phase of mEPSCs was generally well-fitted by a single exponential fit. The kinetics of mEPSCs before and during clonidine application were identical (Fig. 2D). We observed that the amplitude of mEPSCs in 2 of 27 lamina IIo neurons tested was decreased by application of 10 μM of clonidine. The amplitude of mEPSCs of one neuron was decreased from 17.7 to 13.9 pA and another from 34.2 to 23.1 pA.

The effect of clonidine on mIPSCs was tested in 12 lamina IIo neurons in the presence of TTX (1 μM) and CNQX (20 μM). Clonidine (10 μM) did not alter the frequency (0.9 ± 0.3 vs. 0.8 ± 0.3 Hz) and the amplitude (55.1 ± 6.7 vs. 55.3 ± 8.0 pA) of mIPSCs in 10 neurons. In the remaining two neurons, both the frequency and the amplitude of mIPSCs were slightly increased following clonidine application. The frequency of mIPSCs of one neuron was increased from 3.4 to 5.4 Hz and another from 0.1 to 0.2 Hz. The amplitude of mIPSCs of one cell was increased from 34.1 to 56.1 pA and another from 20.2 to 73.9 pA. The mIPSCs were abolished by bath application of bicuculline (10 μM) plus strychnine (5 μM) in all neurons tested.

**Effect of yohimbine and prazosin on clonidine-induced inhibition of mEPSCs**

Yohimbine (2 μM) alone had no effect on the frequency (5.0 ± 0.8 vs. 5.0 ± 0.8 Hz) and the amplitude (18.3 ± 0.9 vs. 17.8 ± 0.9 pA, Figs. 3 and 4) of mEPSCs in 14 lamina IIo neurons. In the presence of yohimbine, clonidine had no effect on the frequency and the amplitude of mEPSCs in these 14 neurons (Figs. 3 and 4). Perfusion with prazosin (2 μM, n = 16) alone did not significantly alter the frequency (4.9 ± 1.0 vs. 4.8 ± 1.0 Hz) and the amplitude (18.6 ± 1.5 vs. 18.3 ± 1.4 pA, Figs. 5 and 6) of mEPSCs. In the presence of prazosin, application of 10 μM of clonidine still significantly decreased
the frequency of mEPSCs from 4.9 ± 1.0 to 2.3 ± 0.6 Hz (P < 0.05, Figs. 5 and 6) but did not significantly change the amplitude (18.6 ± 1.5 vs. 18.4 ± 1.2 pA) and the decay time constant (1.8 ± 0.1 vs. 1.9 ± 0.1 ms) of mEPSCs. The cumulative probability analysis of mEPSCs suggested that the distribution pattern of the inter-event interval (Fig. 5C) shifted toward right, while the amplitude distribution (Fig. 5B) was not altered by clonidine in the presence of prazosin.

Effect of clonidine on eEPSCs of lamina IIo neurons

The eEPSCs were recorded from eight lamina IIo neurons before and after application of 1, 5, 10, and 20 μM of clonidine. The peak amplitude of eEPSCs was attenuated by clonidine in a concentration-dependent fashion (Fig. 7). Clonidine at 10 μM concentration produced a maximal inhibitory effect on the peak amplitude of eEPSCs by 68.4 ± 5.6% (P < 0.05, Fig. 7), compared with the control. In five separate lamina IIo neurons, 2 μM of yohimbine alone did not significantly alter the peak amplitude of eEPSCs. However, perfusion of 10 μM of clonidine failed to alter significantly the amplitude of eEPSCs in the presence of yohimbine (Fig. 8). The eEPSCs were abolished by perfusion of 20 μM of CNQX in five lamina IIo neurons tested (Fig. 8A).

D I S C U S S I O N

Spinal α2-adrenergic receptors mediate a number of physiological functions and pharmacological actions including analgesia. In the present study, we investigated the effect of an α2-adrenergic receptor agonist, clonidine, on the excitatory glutamatergic synaptic input to lamina IIo neurons of the spinal cord. We found that clonidine significantly decreased the frequency of mEPSCs of all lamina IIo neurons tested, but it did not significantly alter the amplitude and the decay time constant of mEPSCs in most (25/27) neurons. The inhibitory effect of clonidine on the mEPSCs of the lamina IIo neurons was abolished by yohimbine but not by prazosin. Furthermore, clonidine attenuated the amplitude of EPSCs of lamina IIo neurons evoked by stimulation of primary afferents in a concentration-dependent manner, and the inhibitory effect of clonidine on eEPSCs of lamina IIo neurons was completely blocked by yohimbine. Therefore these electrophysiological data provide important new evidence that clonidine inhibits the excitatory glutamatergic input to spinal lamina IIo neurons through activation of presynaptic α2-adrenergic receptors. This study also suggests that one of the important physiological functions of spinal α2-adrenergic receptors is to serve as presynaptic heteroreceptors to regulate glutamate release onto lamina IIo neurons from glutamatergic afferent nerve terminals.

Activation of α2-adrenergic receptors in the spinal dorsal horn plays an important role in the inhibition of dorsal horn neurons and analgesia produced by stimulation of the descending noradrenergic system and by intrathecal clonidine (Budai et al. 1998; Nuseir and Proudfoot 2000; Peng et al. 1996; Reddy
Previous studies have demonstrated that stimulation of noradrenergic neurons in the brain stem and pons produces antinociception, which is potentiated by intrathecal α2-adrenergic receptor agonists and blocked by α2-adrenergic receptor antagonists (Hamalainen and Pertovaara 1995; Yeomans et al. 1992). Spinally administrated α2-adrenergic receptor agonists also produce an antinociceptive action and have been used clinically to treat intractable pain conditions (De Kock et al. 1997; Rauck et al. 1993). The mechanisms underlying the analgesic actions of the α2-adrenergic agonists are still not fully known. Previous studies have shown that clonidine produces analgesic actions through the release of other inhibitory neurotransmitters including nitric oxide and acetylcholine in the spinal cord (Pan et al. 1998, 1999). Furthermore there are several lines of investigation supporting the notion that clonidine may produce analgesia through inhibition of the glutamatergic synaptic transmission in the spinal dorsal horn. First, glutamate is involved in nociceptive transmission from primary afferent nerves to superficial dorsal horn neurons (Yoshimura and Jessell 1990), and intrathecal injection of CNQX produces a potent analgesic effect in rats (Chen et al. 2000). Second, it has been demonstrated that the primary localization of the α2A-adrenergic receptors in the rat spinal cord is on the terminals of capsaicin-sensitive C-fiber afferent nerves (Stone et al. 1998). Third, clonidine dose-dependently reduces excitation of dorsal horn neurons evoked by C-fiber stimulation in a yohimbine-reversible manner (Sullivan et al. 1987). Because all mEPSCs recorded from lamina IIo neurons were blocked by CNQX, the mEPSCs represent the quantal release of glutamate from the presynaptic terminals. In the present study, we found that clonidine significantly reduced the frequency of mEPSCs but did not alter the amplitude and kinetics of mEPSCs in most lamina IIo neurons. Because the effect of clonidine was antag-

**FIG. 3.** Effect of yohimbine on the inhibitory effect of 10 μM of clonidine on the mEPSCs of a lamina IIo neuron. **A:** raw traces showing mEPSCs during control, application of 2 μM of yohimbine, and application of 2 μM of yohimbine plus 10 μM clonidine. **B:** cumulative plot analysis of mEPSCs of the same neuron showing the distribution of the peak amplitude during control, yohimbine alone, and application of yohimbine plus clonidine. **C:** cumulative probability plot showing the distribution of the inter-event interval of this neuron during control, yohimbine application alone, and the application of yohimbine plus clonidine. **D:** relative changes of mEPSCs from the control during application of yohimbine plus clonidine.
norepinephrine signiﬁcant inhibits glutamate release onto lamina IIo neurons. In the preliminary study, we also have found evidence that clonidine, through activation of α2-adrenergic receptors located at the glutamatergic nerve terminals, in- 
hibits glutamate release onto lamina IIo neurons.

There are three sources of glutamate release from the pre- 
synaptic nerve terminals in the spinal cord. Activation of the central terminals of primary afferents can cause glutamate release (Yoshimura and Jessell 1990; Yoshimura and Nishi 1993). Glutamate is also a neurotransmitter of the descending inhibitory system and spinal interneurons (Headley and Grill- 
ner 1990). It is difﬁcult to determine the sources of glutamate release and the location of spinal neurons subjected to the inhibition by clonidine from previous neurochemistry experi- 
ments. In the present study, we further determined the effect of clonidine on EPSCs evoked by electrical stimulation of pri- 
mary afferents. Although not examined in this study, the stim- 
ulation parameters used likely stimulated both A- and C-fiber afferents (Yoshimura and Jessell 1989; Yoshimura and Nishi 1993). It has been demonstrated that the lamina IIo neurons receive predominantly C-fiber afferent input (Woodbury et al. 2000). Because all our recordings were made in lamina IIo neurons, it is plausible that the eEPSCs recorded from lamina IIo neurons were caused mainly by C-fiber activation. We observed that clonidine produced a profound inhibitory effect on eEPSCs in a concentration-dependent manner. Because CNQX blocked eEPSCs and the inhibitory effect of clonidine was completely antagonized by yohimbine, these data suggest that the major site of the action of clonidine likely is the α2-adrenergic receptors on the presynaptic terminals of pri- 
mary afferents. Using the blind patch-clamp technique in the 
rat spinal cord slice, Baba et al. (2000) recently reported that norepinephrine causes GABA release through presynaptic α1- 
adrenergic receptors, but it has no effect on the frequency of mEPSCs of lamina II neurons. This finding is unexpected and appears to be different from previous neurochemistry studies (Kamisaki et al. 1993; Ueda et al. 1995) and our own obser-

vation in the present study. The location of the neurons re-
corded from the substantial gelatinosa (not restricted to the lamina IIo) likely accounts for the major difference of these results. Using transganglionic tracers speciﬁc for myelinated and unmyelinated ﬁbers, it has been demonstrated that the myelinated and unmyelinated primary afferents occupy discrete nonoverlapping regions of the lamina II. While the projec-
tion of unmyelinated afferents is restricted to lamina IIo, the 
myelinated afferents occupy only the inner zone of lamina II (lamina II) (Woodbury et al. 2000). Thus it is likely that only the spinal lamina IIo is devoted to nociceptive transmission of C-fiber afferents. In support of this neuroanatomical ﬁnding, we found that clonidine only had a consistent inhibitory effect on mEPSCs recorded from lamina IIo neurons and that clonidine had little effect when recordings were performed on lamina II neurons (data not shown). Our study is consistent with the immunocytochemistry study showing that the location of the α2A-adrenergic receptor subtype is predominantly on the primary C-ﬁber afferents (Stone et al. 1998), which project to lamina IIo neurons (Woodbury et al. 2000). In this regard, data from our electrophysiology study provide further evidence for the functional duality of the lamina II neurons proposed in previous studies (Cervero and Iggo 1980; Woodbury et al. 2000).

We acknowledge that many neuropeptides such as substance P may interact with glutamate on lamina II neurons. However, we believe that it is unlikely that substance P mediates eEPSCs and mEPSCs recorded from lamina II neurons in our study. This is because the eEPSCs and mEPSCs recorded in this study were completely eliminated by 10–20 μM CNQX, and this finding is consistent with previous studies using similar tech-
niques (Yang et al. 2000; Yoshimura and Nishi 1993; Yo-
shimura et al. 1993). Although substance P may mediate slow excitatory postsynaptic potentials (EPSPs) in some lamina IV/V neurons (Yoshimura et al. 1993), substance P application does not evoke any current or affect mEPSCs in spinal lamina II neurons (Yang et al. 2000). Also, it has been shown that application of an NK1 antagonist has no noticeable effect on mEPSCs recorded from lamina II neurons (Yang et al. 2000). Furthermore, a recent study suggests that...
only repetitive stimulation at high intensity (18 V, 0.4 ms, 10–50 Hz) can evoke a small residual EPSC mediated by substance P in some superficial dorsal horn neurons in young rats (Li and Zhuo 2001).

GABA released from spinal inhibitory neurons can access presynaptic GABA \(_B\) receptors (Chery and De Koninck 2000). In a recent study, it has been reported that norepinephrine induces GABA release from the terminals of spinal inhibitory interneurons (Baba et al. 2000). Because presynaptic GABA \(_B\) receptors can modulate glutamate release from the primary afferent terminals in the spinal cord (Iyadomi et al. 2000), clonidine may activate GABAergic interneurons to release GABA, which reduces glutamate release onto lamina IIo neurons indirectly through presynaptic GABA \(_B\) receptors. In the present study, we found that although clonidine increased the frequency and amplitude of mIPSCs in 2 of 12 lamina IIo neurons, it had no effect on mIPSCs in a majority of neurons tested. Thus it is less likely that the effect of clonidine on glutamate release onto lamina IIo neurons is mediated indirectly by the GABA release from inhibitory interneurons. Our data are consistent with a previous study, which demonstrates that clonidine does not alter the GABA release, measured by the HPLC, from spinal synaptosomes (Kamisaki et al. 1993). Because clonidine had little effect on mIPSCs, we did not further explore specifically the effect of clonidine on mIPSCs mediated by glycine and GABA in this study.

Clonidine can bind to imidazoline receptors as well as to \(\alpha_2\)-adrenergic receptors (Ernsberger et al. 1987; Kamisaki et al. 1990). We found that yohimbine, a specific \(\alpha_2\)-adrenergic receptor antagonist devoid of imidazoline structure, completely eliminated the inhibitory effect of clonidine on mEPSCs and eEPSCs. Thus the effect of clonidine on glutamate release onto lamina IIo neurons is mediated by \(\alpha_2\)-adrenergic, but not imidazoline, receptors. The \(\alpha_2\)-adrenergic receptors are pharmacologically classified into \(\alpha_{2A}\), \(\alpha_{2B}\), and \(\alpha_{2C}\)-adrenergic subtypes (Bylund et al. 1988; Lanier et al. 1991). The role of \(\alpha_2\)-adrenergic receptor subtypes involved in the analgesic effect of \(\alpha_2\)-adrenergic agonists remains unclear. In the present study, yohimbine, a nonselective \(\alpha_2\)-adrenergic receptor antagonist, abolished the effect of clonidine on both mEPSCs and mIPSCs and

![FIG. 5. Effect of prazosin on the inhibitory effect of clonidine on mEPSCs of a lamina IIo neuron. A: original tracings showing mEPSCs during control, application of 2 \(\mu\)M of prazosin, and 2 \(\mu\)M of prazosin plus 10 \(\mu\)M clonidine. B: cumulative plot analysis of mEPSCs of the same neuron showing the distribution of the peak amplitude during control, application of prazosin alone, and application of prazosin plus clonidine. C: cumulative probability plot showing the distribution of the inter-event intervals of this neuron during control, application of prazosin alone, and application of prazosin plus clonidine. D: relative changes of mEPSCs from the control during application of prazosin plus clonidine.](http://jn.physiology.org/)


We were unable to further determine the subtypes of $\alpha_2$-adrenergic receptors involved in the effect of clonidine because highly selective $\alpha_2$-adrenergic receptor antagonists are still not available. Importantly, prazosin has been considered to be an $\alpha_2$- and $\alpha_2$-non-A adrenergic receptor antagonist (Bylund 1988; Yaksh et al. 1995). Furthermore, the $\alpha_{2A}$ subtype is the predominant adrenergic receptor located presynaptically in the spinal dorsal horn (Stone et al. 1998). Therefore it is likely that the inhibitory effect of clonidine on glutamate release from primary afferents is mediated by the $\alpha_{2A}$-adrenergic receptor subtype. It should be noted that the $\alpha_2$-adrenergic receptor agonists also could hyperpolarize the dorsal horn neurons through a postsynaptic action (North and Yoshimura 1984). Because $\alpha_2$-adrenergic receptors are located both pre- and postsynaptically in the spinal dorsal horn (Rosin et al. 1993; Shi et al. 1999; Stone et al. 1998), inhibition of eEPSCs by clonidine may be partially mediated by its action on postsynaptic action on $\alpha_2$-adrenergic receptors in lamina II neurons. We observed that the amplitude of the mEPSCs of 2 of 27 lamina II neurons was attenuated by clonidine, suggesting that clonidine may inhibit a few lamina II neurons through a postsynaptic action.

In summary, this electrophysiological study provides new information that clonidine inhibits the excitatory synaptic input to spinal lamina II neurons by activation of presynaptic $\alpha_2$-adrenergic receptors. We were unable to further determine the subtypes of $\alpha_2$-adrenergic receptors involved in the effect of clonidine because highly selective $\alpha_2$-adrenergic receptor antagonists are still not available. Importantly, prazosin has been considered to be an $\alpha_2$- and $\alpha_2$-non-A adrenergic receptor antagonist (Bylund 1988; Yaksh et al. 1995). Furthermore, the $\alpha_{2A}$ subtype is the predominant adrenergic receptor located presynaptically in the spinal dorsal horn (Stone et al. 1998). Therefore it is likely that the inhibitory effect of clonidine on glutamate release from primary afferents is mediated by the $\alpha_{2A}$-adrenergic receptor subtype. It should be noted that the $\alpha_2$-adrenergic receptor agonists also could hyperpolarize the dorsal horn neurons through a postsynaptic action (North and Yoshimura 1984). Because $\alpha_2$-adrenergic receptors are located both pre- and postsynaptically in the spinal dorsal horn (Rosin et al. 1993; Shi et al. 1999; Stone et al. 1998), inhibition of eEPSCs by clonidine may be partially mediated by its action on postsynaptic action on $\alpha_2$-adrenergic receptors in lamina II neurons. We observed that the amplitude of the mEPSCs of 2 of 27 lamina II neurons was attenuated by clonidine, suggesting that clonidine may inhibit a few lamina II neurons through a postsynaptic action.

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adrenergic receptors located on the glutamatergic afferent terminals. Our data strongly suggest that the spinal α2-adrenergic receptors on the primary C-fiber afferent terminals function as heteroreceptors to regulate glutamate release onto lamina II o neurons. These findings are important for our understanding of the mechanisms of the analgesic actions produced by spinal α2-adrenergic receptor agonists. The presynaptic inhibitory control of glutamate release by spinal α2-adrenergic receptors also may be important for the inhibition of nociceptive transmission in the spinal lamina II o neurons by the descending noradrenergic system.

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