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Modulation of inhibitory synaptic activity by a non-α4β2, non-α7 subtype of nicotinic receptors in the substantia gelatinosa of adult rat spinal cord

Daisuke Takeda[a,b], Terumasa Nakatsuka[a,b], Roger Papke[b,c,d], Jianguo G. Gu[a,b,*]

[a]*Department of Oral Surgery, College of Dentistry, University of Florida, 1600 SW Archer Road, Gainesville, FL 32610, USA*
[b]*Evelyn F. & William L. McKnight Brain Institute of the University of Florida, Gainesville, FL 32610, USA*
[c]*Department of Pharmacology, College of Medicine, University of Florida, Gainesville, FL 32610, USA*
[d]*Department of Neuroscience, College of Medicine, University of Florida, Gainesville, FL 32610, USA*

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Abstract

The GABA/glycine-mediated inhibitory activity in the substantia gelatinosa (SG) of the spinal cord is critical in the control of nociceptive transmission. We examined whether and how SG inhibitory activity might be regulated by neuronal nicotinic receptors (nAChRs). Patch-clamp recordings were performed in SG neurons of spinal slice preparations from adult rats. We provided electrophysiological evidence that inhibitory presynaptic terminals in the SG expressed nAChRs and their activation resulted in large increases in the frequency of spontaneous and miniature inhibitory postsynaptic currents (sIPSCs and mIPSCs) in over 90% SG neurons tested. The enhancement of inhibitory activity was mediated by increases in the release of GABA/glycine, and direct Ca^{2+} entry through SG presynaptic nAChRs appeared to be involved. Miniature IPSC frequency could be enhanced by the nAChR agonists nicotine or cytisine. Nicotine could still elicit large increases in mIPSC frequency in the presence of the α4β2 nAChR antagonist dihydro-beta-erythroidine (5 μM) and the α7 nAChR-selective antagonist methyllycaconitine (40 nM). However, nicotine did not produce a significant enhancement of mIPSC frequency in the presence of the broad spectrum nAChR antagonist mecamylamine (5 μM). Nicotinic agonist-evoked whole-cell currents from SG neurons and the antagonist profiles also indicated the presence of a subtype of nAChRs, which were different from the major central nervous system nAChR subtypes, i.e. α4β2* or α7 nAChRs. Together, our results suggest that a subtype of nAChR, possibly α3β4* nAChR or a new nAChR type, is highly expressed at the inhibitory presynaptic terminals in SG of adult rats and play a role in the control of inhibitory activity in SG.

Keywords: Nicotinic receptors; Inhibitory postsynaptic currents; Gamma-aminobutyric acid; Glycine; Patch-clamp technique; Spinal cord slice preparation

1. Introduction

Nicotine was reported to have analgesic effects 70 years ago (Davis et al., 1932). Nicotinic agonists could have more potent analgesic effects than morphine in animals (Spande et al., 1992; Sullivan et al., 1994). The sensory regions of both supraspinal structures and spinal cord dorsal horn (DH) appear to be involved in the analgesic actions of nicotinic analogs (Marubio et al., 1999; Aceto et al., 1986; Damaj et al., 1998). At the spinal cord level, efforts are being made to determine the mechanisms by which neuronal nicotinic receptors (nAChRs) may be involved in regulating nociceptive transmission (Cordero-Erausquin and Changeux, 2001).

The substantia gelatinosa (SG) of the spinal cord DH plays an important role in modulating nociceptive transmission (Willis and Coggeshall, 1991). SG is concentrated with interneurons, and many are GABAergic or glycinergic inhibitory neurons. These inhibitory neurons synapse presynaptically on primary afferent terminals and postsynaptically on DH neurons, which provides inhibitory controls in nociceptive pathways (Doubell et al., 1999). A change in the release probability of GABA and glycine in SG may have profound effects on nociceptive transmission.

The release of GABA or glycine in the SG may be regulated by nAChRs (Urban et al., 1989; Cordero-Erausquin and Changeux, 2001). In brain regions, nAChRs that are expressed at preterminals or presynaptic terminals mediate the enhancement of neurotransmitter release (McGehee et al., 1995; Gray et al., 1996; Albuquerque et al., 1997; Wonnacott, 1997; Alkondon et al., 1997, 1999; Guo et al., 1998; Li et al., 1998; Mansvelder and McGehee, 2000; Radcliffe et al., 1999; Barazangi and Role, 2001). nAChRs are formed by subunits of α2-α6 and β2-β4 in α/β combinations or by α7-α9 in homomeric forms (McGehee, 1999;

* Corresponding author. Tel.: +1-352-392-5989; fax: +1-352-392-7609.
E-mail address: jgu@dental.ufl.edu (J.G. Gu).

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Dani, 2001). Although these subunits can form numerous nAChRs in heterologous expression systems, the major central nervous system (CNS) nAChRs were found to be α4β2* (the recommended nomenclature for nAChR subtypes containing α4, β2, and possibly other subunits, Lukas et al., 1999) and α7 nAChRs in most brain regions (McGehee, 1999; Dani, 2001). Alpha4beta2* nAChRs are selectively blocked by low concentrations of dihydro-beta-erythroidine (DhβE) (Albuquerque et al., 1997); (*E*)-*N*-methyl-4-(3-pyridinyl)-3-butene-1-amine (RJR-2403) has been found to be a selective agonist (Papke et al., 2000). Alpha7 nAChRs show high sensitivity to blockade by α-bungarotoxin and methyllycaconitine (MLA) (Ward et al., 1990; McGehee et al., 1995; Mansvelder and McGehee, 2000); choline is a selective agonist (Papke et al., 1996). Consistent with these pharmacological profiles, preterminal or presynaptic nAChRs appeared to be α4β2* or α7 nAChRs in most brain regions (Lena et al., 1993; McMahon et al., 1994b; McGehee et al., 1995; McGehee, 1999).

In the spinal cord, mRNAs for most nAChR subunits have been identified during early developmental stages (Wada et al., 1989, 1990; Hellstrom-Lindahl et al., 1998). Studies in postnatal rats showed that nicotinic analogs had excitatory effects on DH neurons in deep laminae (Urban et al., 1989; Bordey et al., 1996). Behavioral studies showed that intrathecal application of nicotinic agonists could elicit both analgesic and algesic effects (Khan et al., 1998, 2001). Recently, it has been shown that nAChRs play a role in modulating serotonin release in the spinal cord (Cordero-Erausquin and Changeux, 2001) and modulating glutamate release from primary afferent terminals in DH (Genzen and McGehee, 2000). More recently, Kiyosawa et al. (2001) demonstrated that, in postnatal rats, nicotinic agonists facilitated glycine release in the spinal cord DH neurons through the activation of α4β2* subtype of nAChRs. Here, we examined, in the SG of spinal slices from adult rats, the expression of nAChRs and their agonist and antagonist profiles with patch-clamp recordings. We determined the role of these receptors in modulating GABA/glycine release.

2. Methods

2.1. Tissue preparation

Transverse spinal cord slices (600 μm in thickness) were prepared from L5 spinal cords of adult Sprague–Dawley rats (Harlan, IN, USA) aged between 6 and 9 weeks (250–400 g). In brief, rats were continuously anaesthetized with isoflurane through nose cone inhalation delivered by an Isoflurane-anaesthetizing machine. A lumbosacral laminectomy was performed. The lumbosacral segment of the spinal cord (L1-S3) was rapidly cut out and placed in ice cold (1–3°C) Krebs solution pre-equilibrated with 95% O_2 and 5% CO_2. The Krebs solution contained (in mM): NaCl, 117; KCl, 3.6; $CaCl_2$, 2.5; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 11. After cutting off ventral and dorsal roots near the root entry zone, the pia-arachnoid membrane was completely removed. The spinal cord was fixed on an agar block and mounted on a Vibratome, and was cut into 600-μm slices.

A spinal cord slice was transferred to a recording chamber (volume of 0.5 ml). The slice was supported at the bottom by a nylon mesh in the recording chamber. A platinum grid was placed on the top of the slice to prevent slice movement (Fig. 1). The slice was completely submerged in and superfused with Krebs solution at flow rate of 10 ml/min. The Krebs solution was equilibrated with 95% O_2 and 5% CO_2 and maintained at room temperature (22°C); pH of the solution was 7.35. Lamina regions were identified under a dissecting microscope with 40× magnification based on morphological features. The SG was clearly discernible as a relatively translucent band across the superficial DH (Fig. 1). Because the border between laminae I and lamina II, and also that between laminae II and III could not be determined with certainty, the patch electrode was inserted vertically at the center of the SG under visual guidance.

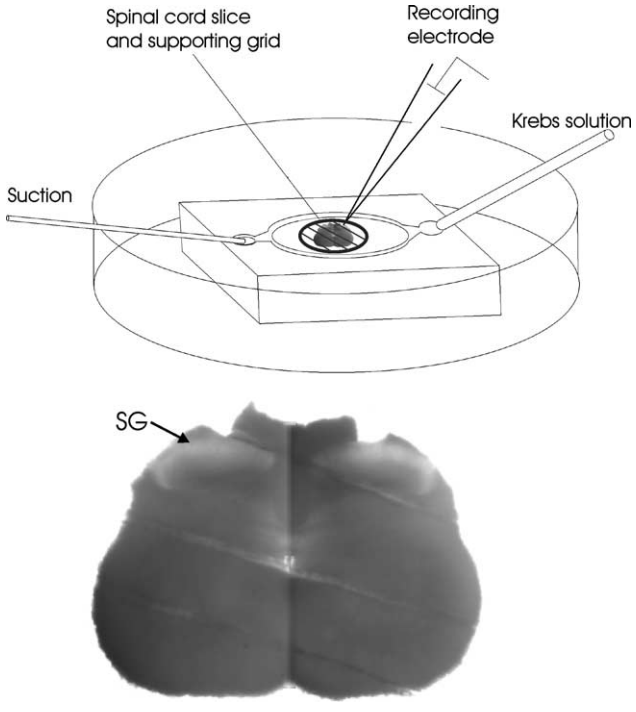

Fig. 1. Blind-patch recordings from substantia gelatinosa in spinal cord slice preparations from adult rats. The diagram on the top panel shows part of recording setup. The bottom image is a L5 spinal slice from an adult rat at the age of 55 days. Under a dissecting microscope with transmitted light, the substantia gelatinosa (SG, indicated with an arrow) was clearly discernible as a relatively translucent band across the superficial DH. Over 200 cells were recorded in this study. Of these cells, cell capacitance and membrane resistance were measured in 102 cells, they were 25 ± 1 pF and 360 ± 34 MΩ, respectively.

2.2. Patch-clamp recordings

Blind-patch technique was applied in all experiments. Cell patch was usually made 150–300 μm below the surface of the slice. Whole-cell patch-clamp recordings were made from DH neurons in the SG with electrodes (~8 MΩ) filled with a solution containing (in mM): Cs_2SO_4, 110; $CaCl_2$, 0.5; $MgCl_2$, 2; Tea-Cl, 5; EGTA, 5; HEPES, 5; pH adjusted with NaOH to 7.2. Signals were amplified and filtered at 2 kHz (Axopatch 200B) and sampled at 5 kHz. When spontaneous inhibitory postsynaptic currents (sIPSCs) or miniature inhibitory postsynaptic currents (mIPSCs) were recorded, cells were held at −10 mV. Spontaneous IPSCs were recorded in the absence of tetrodotoxin (TTX). In one set of experiments, sIPSCs were recorded in a bath solution containing 20 μM 6-cyano-7-nitroquinoxalin-2,3-dione (CNQX) plus 50 μM D-(−)-2-amino-5-phosphonovaleric acid (APV). Isolation of GABAergic or glycinergic sIPSCs was accomplished by including 2 μM strychnine or 20 μM bicuculline in the bath solution. Miniature IPSCs were recorded in the presence of 0.5 μM TTX. In one set of experiments, mIPSCs were recorded in the presence of 30 μM $LaCl_3$ as well as 0.5 μM TTX. When spontaneous excitatory postsynaptic currents (sEPSCs) were recorded, cells were held near −50 mV. When nicotinic agonist-evoked whole-cell currents were examined in SG neurons, cells were held at −60 mV, and the bath solution contained 20 μM CNQX, 50 μM APV, 20 μM bicuculline, and 2 μM strychnine. Nicotinic agonists tested in this study include nicotine, cytisine, choline, and RJR-2403. Nicotinic antagonists tested include mecamylamine (Mec), MLA, and DhβE. All compounds tested were applied through the bath solution at a flow rate of 10 ml/min. All nicotinic antagonists and blockers for other ion channels were preapplied for at least 10 min at a flow rate of 10 ml/min. Analysis of sIPSCs, mIPSCs, and sEPSCs, including threshold setting and peak identification criteria, were performed according to a method previously described (Gu et al., 1996; Gu and MacDermott, 1997). Unless otherwise indicated, each recording was performed on a cell in a fresh slice without prior application of any agonist or antagonist.

DhβE, cytisine, MLA, Mec, bicuculline, strychnine, choline, and $LaCl_3$ were purchased from Sigma (St. Louis, MO, USA). CNQX, D-APV, TTX, RJR-2403 were purchased from Tocris (St. Louis, MO, USA). Nicotine was purchased from RBI (Natick, MA, USA). Unless otherwise indicated, data represent Mean ± SEM, Student's *t*-tests were used for statistical comparison, and significance was considered at the $P < 0.05$ level.

3. Results

We used adult rats in all our experiments to avoid complications due to different nAChR subtype expression during development (Wada et al., 1989; Zoli et al., 1995; Hellstrom-Lindahl et al., 1998). We first determined whether nicotine could enhance inhibitory synaptic activity in SG neurons. This was done by recording sIPSCs. The sIPSCs had a reversal potential near −50 mV (Fig. 2A). When cells were held at −10 mV, all sIPSCs were shown to be outward currents, and inward glutamatergic sEPSCs were minimized (Fig. 2B). The outward synaptic currents were GABAergic/glycinergic sIPSCs as they could be completely blocked following the inclusion of 20 μM bicuculline plus 2 μM strychnine (Fig. 2B). Nicotine (100 μM) produced robust increases in the frequency of sIPSCs (Fig. 2C) in almost all SG neurons recorded. Of 24 cells tested with 100 μM nicotine, 23 cells (96% of cells) responded following the bath application of nicotine for 1 min, one cell did not show a detectable change of sIPSC frequency. The overall increase of the sIPSC frequency following nicotine applica-

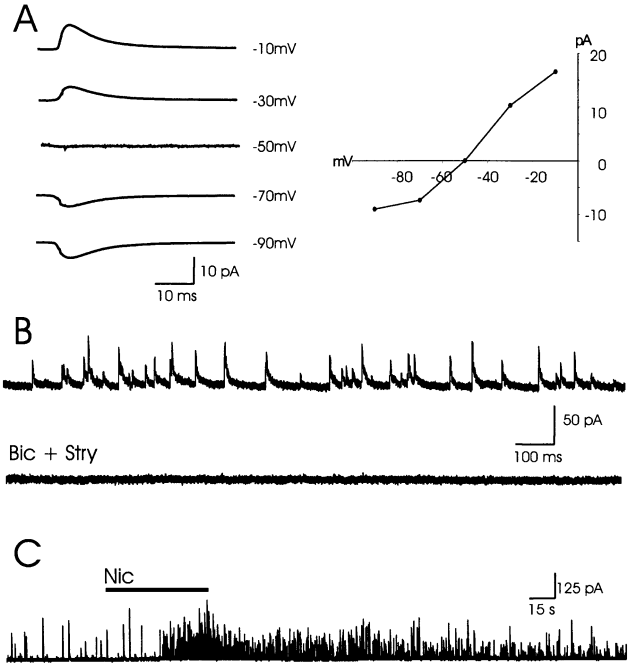

Fig. 2. Nicotine-induced increases of spontaneous inhibitory synaptic activity in substantia gelatinosa neurons. (A) Left traces show spontaneous inhibitory synaptic currents (sIPSCs) recorded from a SG neuron at different holding potentials. Each sIPSC trace was the average of IPSCs (~200 events) in a 1-min period of recording at a holding potential. The bath solution contained 20 μM CNQX and 50 μM AVP. The data is also plotted as an I–V curve (right panel) to show a reversal potential of about −50 mV. (B) An example shows outward sIPSCs recorded from a SG neuron held at −10 mV (top trace), which was blocked completely by 20 μM bicuculline plus 2 μM strychnine (bottom trace). (C) An example shows a large increase in sIPSC frequency following bath application of 100 μM nicotine. The time period of nicotine application is indicated by a horizontal bar above the trace. The experiments in (C) were performed in the absence of CNQX and APV. Of a total of 24 cells tested, 23 cells showed increase in mIPSC (miniature inhibitory postsynaptic current) frequency by 100 μM nicotine. The changes of mIPSC frequency in these cells are illustrated in Fig. 3C for comparison with other results shown there. In experiments in B,C and other experiments below for determining sIPSCs or mIPSCs, cells were held at −10 mV. At this holding voltage, potential effects of nicotine on postsynaptic cells, i.e. nicotine-induced whole-cell currents and nAChR channel noise, were minimized during nicotine application.

tion was 1017 ± 176% of control, from 13 ± 3 Hz in control conditions to 118 ± 30 Hz after nicotine application ($n = 24$, $P < 0.05$, also see Fig. 3C). Nicotine at 10 μM also increased sIPSC frequency ($n = 3$, data not shown).

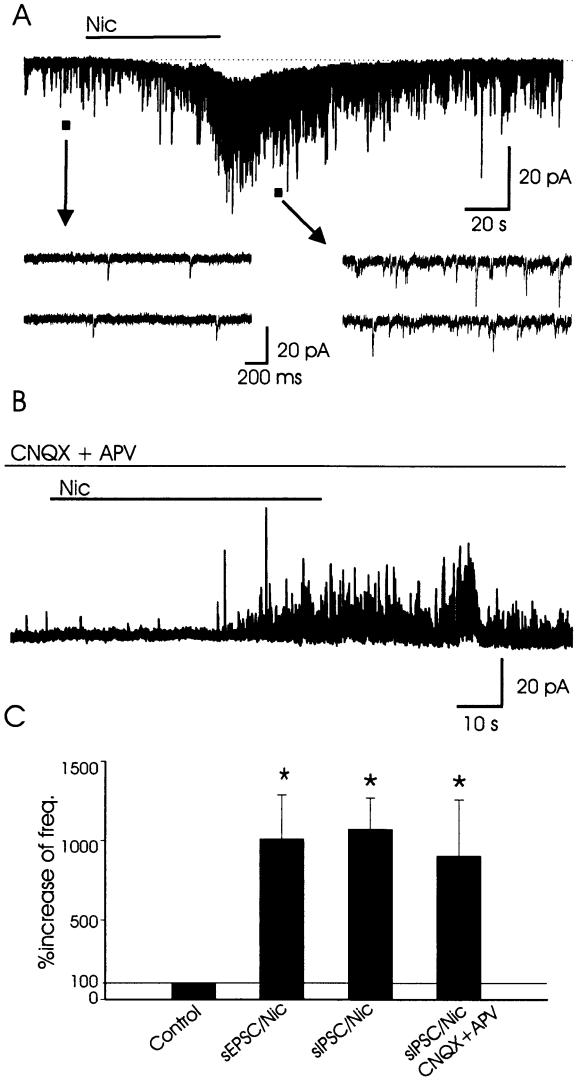

Fig. 3. Most nicotinic effects on inhibitory synaptic activity are not associated with nicotine-induced increases of excitatory glutamatergic activity. (A) Nicotine produced a substantial increase of sEPSC frequency in SG neurons. The top trace shows continuous recording of sEPSCs before and after bath application of 100 μM nicotine. There was a large increase of sEPSC frequency, which can be seen clearly in the expanded scale in the bottom two sets of traces. An inward current was also seen following the application of 100 μM nicotine (top trace), which was unlikely due to the summation of sEPSCs (right panel of bottom 2 traces). Similar results were observed in the other two cells. In all experiments, sIPSC reversal potentials were first examined and then sEPSCs were recorded at sIPSC reversal potentials (−50 to −60 mV). (B) Nicotine produced an increase in sIPSC frequency following the blockade of glutamatergic synaptic activity with 20 μM CNQX plus 50 μM APV. In all experiments, cells were held at −10 mV. (C) Summary of nicotine-induced increase of sEPSC frequency ($n = 3$), and nicotine-induced increases of IPSC frequency in the absence ($n = 24$) and presence of 20 μM CNQX plus 50 μM APV ($n = 6$). Data represent Mean ± SEM. *$P < 0.05$, comparing with control.

Nicotine may increase the release of glutamate from primary afferent terminals and/or from spinal cord excitatory neurons, which in turn excite spinal inhibitory neurons. Therefore, one possibility for the increases of sIPSC frequency in SG neurons following nicotine applications might be due to effects on the nAChRs located on primary afferent terminals (Genzen and McGehee, 2000) and/or spinal cord excitatory neurons. Recordings of sEPSCs showed that nicotine (100 μM) could indeed increase the frequency of sEPSCs (1009 ± 280%, $n = 3$, Fig. 3A). However, when CNQX (20 μM) and APV (50 μM) were included in the bath solution to prevent the potential glutamatergic driving activity, 100 μM nicotine still produced a large increase in sIPSC frequency (Fig. 3B). The increases of sIPSC frequency in the presence of 20 μM CNQX and 50 μM APV were 901 ± 357% of control ($n = 6$, $P < 0.05$, Fig. 3C). The degree of the increases under this condition was not significantly different from that in the absence of the two antagonists (901 ± 357 vs 1017 ± 176%, Fig. 3C). These results suggest that most, if not all, of the effect of nicotine on the inhibitory synaptic activity in the SG is not associated with its effect on the excitatory pathways.

sIPSCs recorded from SG neurons were of two kinetically different types: one had slow decay rates, and the other type had rapid decay rates (Fig. 4A). In the presence of the glycine receptor inhibitor, 2 μM strychnine, the slow kinetic type of sIPSCs was observed exclusively, indicating that they were GABAergic sIPSCs (Fig. 4A). On the other hand, in the presence of the GABA(A) receptor inhibitor, 20 μM bicuculline, the fast kinetic type of sIPSCs was observed, indicating that they were glycinergic sIPSCs (Fig. 4A). We examined whether nicotine could increase GABAergic sIPSC frequency in the presence of 2 μM strychnine. Under this condition, 100 μM nicotine increased sIPSC frequency (1381 ± 391% of control, $P < 0.05$) in all the nine cells tested (Fig. 4B). We also tested whether nicotine could produce an increase of glycinergic sIPSC frequency by inclusion of 20 μM bicuculline in the bath solution to block GABA(A) receptors. Under this condition, 100 μM nicotine produced large increases of sIPSC frequency as well (1103 ± 322% of control, $n = 9$, $P < 0.05$, Fig. 4C).

We determined whether nAChRs might be localized at inhibitory neuron presynaptic terminals in the SG region and if so, whether their activation could directly regulate the release of GABA/Glycine. This was accomplished by examining the effects of nicotine on mIPSCs in the presence of 0.5 μM TTX, 20 μM CNQX, and 50 μM APV. At the concentrations of TTX, CNQX, and APV used, Na^+ channels and glutamatergic postsynaptic currents were completely blocked in the neurons of our spinal cord preparations (data not illustrated, see also Nakatsuka and Gu, 2001). Under this condition, bath application of nicotine (100 μM) produced increases of mIPSC frequency in most of the cells examined (Fig. 5A, pooled data are illustrated in Fig. 6D, E). Of 17 cells examined, 15 cells showed increases

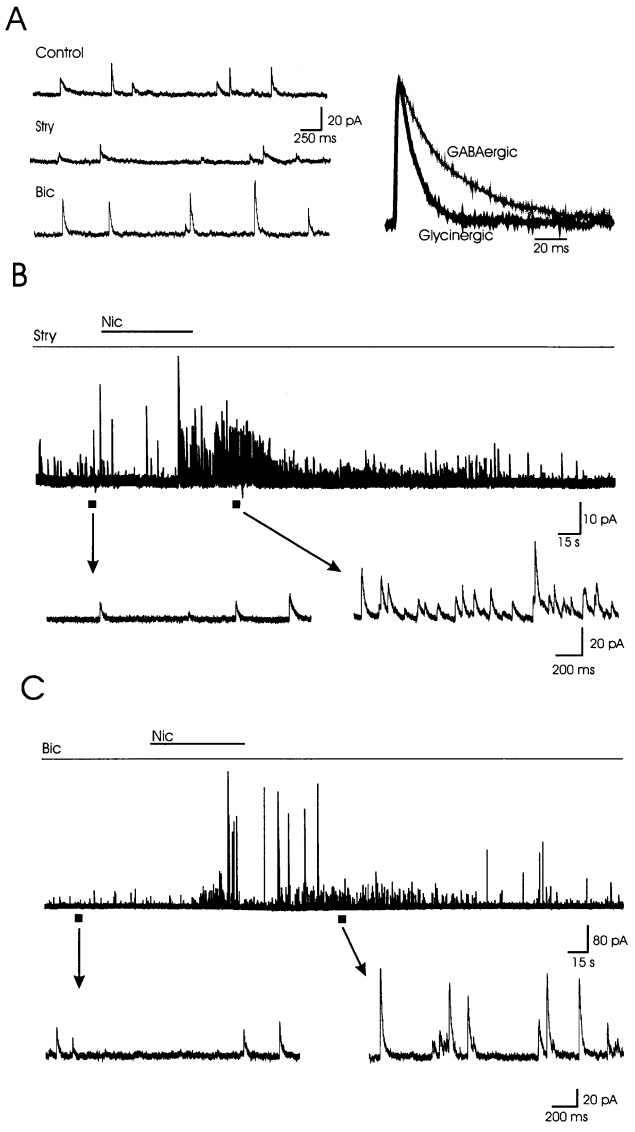

Fig. 4. Nicotine produced increases of both GABAergic and glycinergic sIPSC frequency in SG neurons. (A) Left three traces show sIPSCs in the absence, or in the presence of 2 μM strychnine or 20 μM bicuculline. In the absence of strychnine and bicuculline (top trace), two types of IPSCs, the fast sIPSCs and the slow sIPSCs could be observed. In the presence of strychnine (middle trace), only slow sIPSCs were observed. In the presence of bicuculline (bottom trace), only fast sIPSCs were seen. Two traces on the right panel show different kinetics in decay phases for the sIPSCs in the presence of either strychnine (GABAergic sIPSC) or bicuculline (glycinergic sIPSC). Each trace was the average of sIPSCs in 1 min period of recoding. The averaged sIPSCs shown in the figure were scaled to the same amplitude. (B) Increases of GABAergic sIPSCs by nicotine. The top trace shows continuous recording of sIPSCs in the presence of 2 μM strychnine in a SG neuron. Two traces on the bottom show, in an expanded scale, the sIPSC frequency before and following the application of 100 μM nicotine. Similar effects were observed in other eight cells. (C) Increases of glycinergic sIPSCs by nicotine. The experiments were similar to that in (B) except that sIPSCs were recorded in the presence of 20 μM bicuculline. Similar effects were observed in eight other cells.

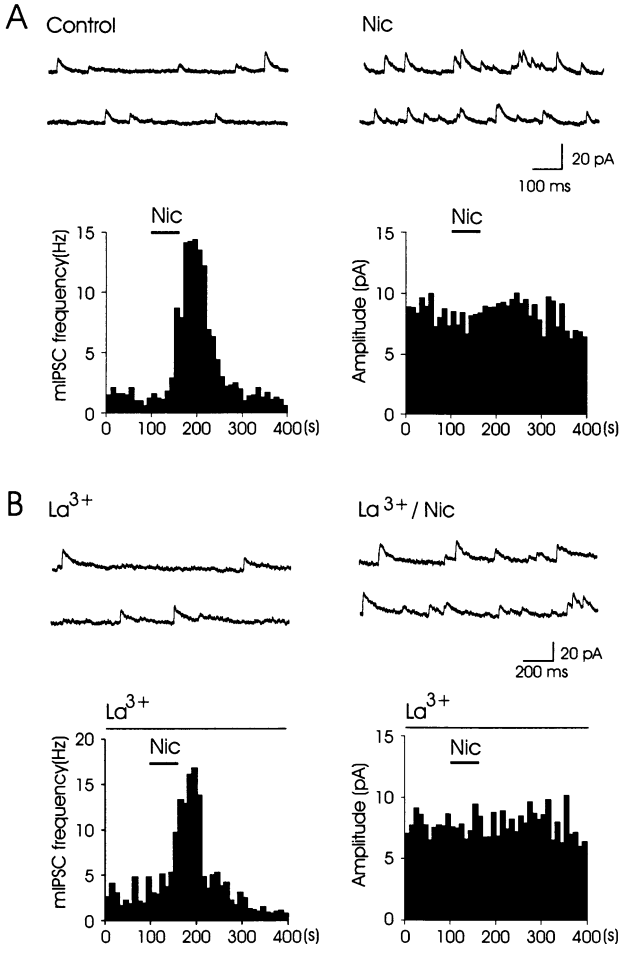

Fig. 5. Nicotine-induced increases of mIPSC frequency. (A) Two sets of sample traces show the mIPSCs before (control) and after bath application of 100 μM nicotine in a SG neuron. Two sets of histograms show the time courses of mIPSC frequency (left) and mIPSC amplitude (right) before and after the application of 100 μM nicotine. Similar results were obtained in 14 other cells. The overall changes of mIPSCs by nicotine are illustrated in Fig. 6D,E for comparison with the effects of other nicotinic agonists. (B) The experiment was similar to that shown in (A) except that the voltage-gated Ca^{2+} channel blocker, 30 μM La^{3+}, was included in the bath solution. Similar results were obtained in six other cells.

of mIPSC frequency (Fig. 6D, E). The overall changes of mIPSC frequency were 1386 ± 23% of control, from 9 ± 1 Hz of control to 90 ± 17 Hz following nicotine application ($n = 17$, $P < 0.05$). While nicotine increased mIPSC frequency, mIPSC amplitude was not significantly changed (102 ± 1.2% of control, $n = 17$, Fig. 5A). The increases of mIPSCs by nicotine, i.e. the increase of GABA/glycine release probability, could be due to the direct Ca^{2+} entry through presynaptic nAChRs or the terminal depolarization-induced Ca^{2+} entry through voltage-gated Ca^{2+} channels. To determine whether Ca^{2+} entry through presynaptic nAChRs was involved in the increases of GABA/glycine release, we examined effects of nicotine on mEPSC frequency in the presence of the voltage-gated Ca^{2+} channel blocker, 30 μM La^{3+} (Gu and MacDermott, 1997). Under this condition, nicotine still produced increases in mIPSC frequency (1002 ± 193% of control, $n = 7$, $P < 0.05$) without affecting mIPSC amplitude (Fig. 5B).

We examined effects of different nicotinic agonists and

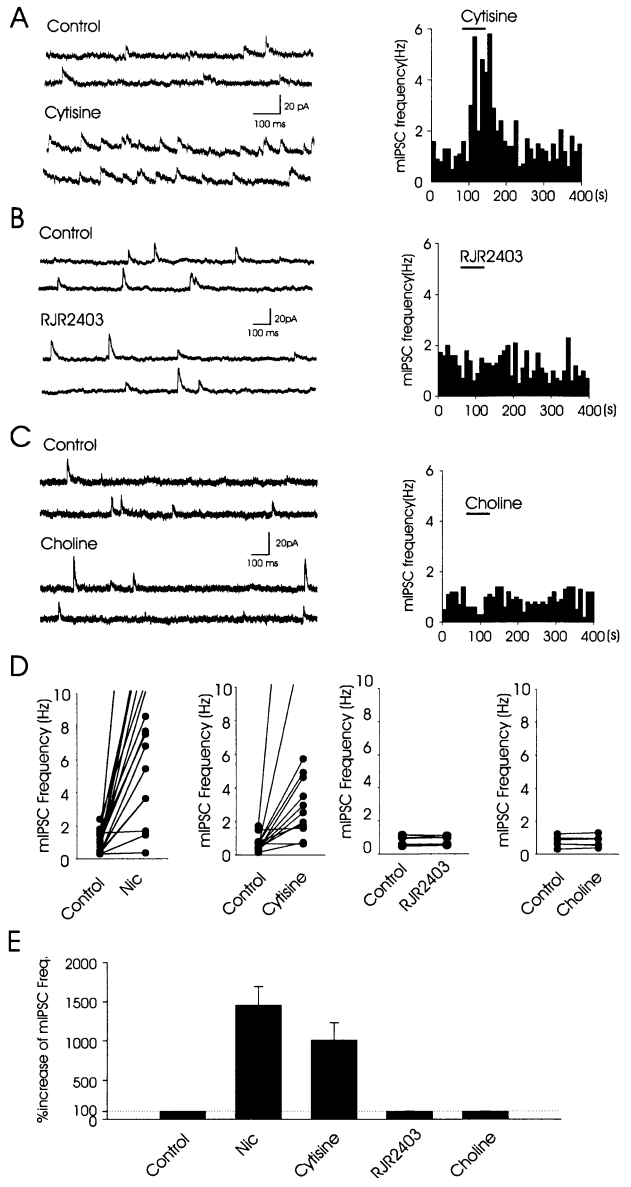

Fig. 6. Effects of different nicotinic agonists on mIPSC frequency. (A) Two sets of sample traces on the left panel show the mIPSCs before and after bath application of 20 μM cytisine. The histogram on the right panel shows the time course of mIPSC frequency before and after the application of cytisine. (B,C) Experiments were similar to that shown in (A) except that 100 μM RJR-2403 or 10 mM choline was tested. (D) Pooled data show the mIPSC frequency before and after the applications of different nicotinic agonists in all individual cells. The first panel shows the responses following 100 μM nicotine ($n = 17$), and sample traces are illustrated in Fig. 5A. The remaining three panels are the responses following 20 μM cytisine ($n = 13$), 100 μM RJR-2403 ($n = 7$), and 10 mM choline ($n = 6$), respectively. Each recording is illustrated by a pair of solid circles connected with a line. Some lines are truncated, as the increases of mIPSC frequency in some cells were very large. (E) The graph shows the changes of mIPSCs, as a percentage of controls, following the application of 100 μM nicotine, 20 μM cytisine, 100 μM RJR-2403, or 10 mM choline.

antagonists that are known to have preferential effects on subtypes of nAChRs. Three agonists, cytisine (20 μM), RJR-2403 (100 μM), and choline (10 mM) were examined to determine their effects on mIPSC frequency in SG neurons. Similar to nicotine, 20 μM cytisine also produced a large increase in mIPSC frequency (Fig. 6A, D, E) without affecting mIPSC amplitude (not shown). Of 13 cells tested with 20 μM cytisine, 11 of them showed responses to cytisine by increases of mIPSC frequency. The overall changes of mIPSC frequency were 1011 ± 224% of control ($n = 13$, $P < 0.05$). On the other hand, RJR-2403 (100 μM) did not affect mIPSC frequency (99 ± 3% of control, $n = 7$, Fig. 6B, D, E). Choline (10 mM) also did not have effect on mIPSC frequency (102 ± 5% of control, $n = 6$, Fig. 6C, D, E). Fig. 6D shows the mEPSC frequency at baseline level (control) and following the application of different nicotinic agonists in each individual cell recorded. A summary of the effects on mIPSC frequency by nicotine and other nicotinic agonists, expressed as percentage of control, is shown in Fig. 6E.

We examined the effects of different nicotinic antagonists on nicotine-induced increases of mIPSC frequency. Because full recovery of nicotinic responses from a prior nicotine application was poor even after prolonged wash in normal bath (not shown), we examined the effects of nicotine alone compared to nicotine plus different antagonists in separate groups of cells to evaluate the potential inhibitory effects on nicotine-mediated responses. This approach is valid in our study because over 90% of neurons responded to nicotine with a large increase of mIPSC frequency (Fig. 6D, E). While nicotine alone produced a 1386 ± 234% increase in mIPSC frequency as shown in Fig. 6D, E, in the presence of 5 μM Mec, 100 μM nicotine was unable to produce any significant changes in mIPSC frequency in any recorded cells (107 ± 4% of control, $n = 8$, Fig. 7A, E and F). On the other hand, in the presence of 5 μM DhβE, 100 μM nicotine could still produce a large increase in mIPSC frequency (Fig. 7B, E, F). Of 12 cells examined, all of them showed nicotine-induced increases in mIPSC frequency (942 ± 258%, $P < 0.05$, Fig. 7E, F). In the presence of MLA (40 μM), 100 nM nicotine also produced a large increase in mIPSC frequency in ten cells out of 11 recorded neurons (Fig. 7C, E, F). The overall changes were 854 ± 238% of control ($n = 11$, $P < 0.05$, Fig. 7F). The use of MLA alone could not exclude the possible contribution of DhβE-sensitive nAChRs to the increases of mIPSC frequency; the use of DhβE alone could not exclude the possible contribution of MLA-sensitive nAChRs. Therefore, we further determined the effects of nicotine in the presence of both MLA (40 nM) and DhβE (5 μM). Under these conditions, nicotine still produced a large increase in the mIPSC frequency (Fig. 7D, E and F). The increases of mIPSC frequency by nicotine were 1434 ± 361% of control in all six cells tested (Fig. 7E, F). In all the above experiments, antagonists alone were not found to significantly change the basal mIPSC frequency.

We directly examined the expression of nAChRs on SG neurons by recording nicotine-evoked whole-cell currents. We further determined their agonist and antagonist profiles

to see whether they are consistent with the profiles observed in mIPSC experiments. Whole-cell currents were recorded at a holding potential of −60 mV in the bath solution containing inhibitor cocktail solutions with 20 μM bicuculline, 2 μM strychnine, 20 μM CNQX and 50 μM APV. Of 14 cells recorded, 13 cells (93%) showed inward whole-cell currents elicited by bath application of 100 μM nicotine. The nicotine-evoked currents had a mean peak amplitude of 33 ± 8 pA and lasted for 287 ± 21 s following a 1 min application of nicotine at flow rate of 10 ml/min (Fig. 8A, I). There was also increased channel-opening noise associated with the whole-cell inward currents at the holding potential of −60 mV. However, the channel-opening noise was not significantly higher than baseline noise at holding potential of −10 mV where sIPSCs and mIPSCs were determined (not shown). Repeated nicotine applications showed an incomplete recovery of responses (Fig. 8B). Recovery after the first nicotine application was less than 40% (37 ± 3%, $n = 4$) even after a 30–60 min wash in the normal bath solution at 10 ml/s flow rate. We examined effects of the other nicotinic agonists cytisine, RJR-2403, and choline on whole-cell currents. Cytisine (20 μM) also evoked inward whole-cell currents in all four cells tested. The mean peak amplitude of cytisine-evoked currents was 38 ± 17 pA and currents lasted for 312 ± 88 s (Fig. 8C, I), and was not significantly different from nicotine-evoked currents ($P = 0.40$). On the other hand, RJR-2403 (100 μM) did not produce any detectable currents in all the other eight cells tested (Fig. 8D, I), significantly different from nicotine-mediated responses ($P < 0.001$). Choline (10 mM) also did not evoke any whole-cell current in all the other four cells tested (Fig. 8E, I), significantly different from nicotine-mediated responses as well ($P < 0.001$).

Because repeated applications of nicotine could not produce the same whole-cell currents in our adult spinal cord slice preparation (Fig. 8B), it is difficult to determine the antagonist profiles on the same neurons. For this reason, all experiments described in this article, except the one shown in Fig. 8B, were performed with a single agonist application on a fresh slice without prior drug application. This approach is valid because over 90% of SG neurons responded to nicotine. In a group of 12 cells for which 5 μM Mec was continuously present (Fig. 8F, I), nicotine was unable to elicit any detectable current in any of these cells ($P < 0.001$, comparing with the responses by nicotine alone). On the other hand, in another group of slices in the presence of 5 μM DhβE, 100 μM nicotine still could elicit inward currents in all eight cells tested (Fig. 8G). The mean

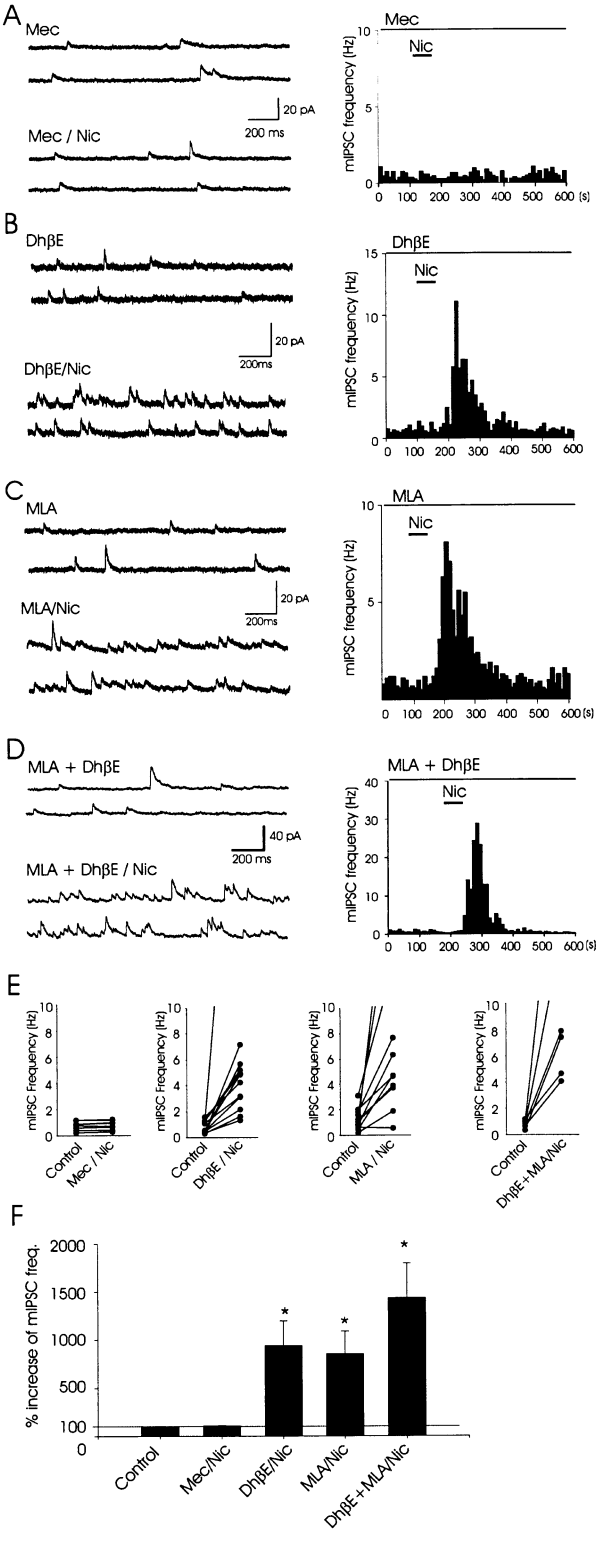

Fig. 7. Antagonist profiles of nicotine-induced increases of mIPSCs. (A–D) Effects on nicotine-induced increases of mIPSCs by the nicotinic antagonists 5 μM mecamylamine (Mec), 5 μM DhβE, 40 nM MLA, or 40 nM MLA plus 5 μM DhβE. In the presence of 5 μM Mec (A), 100 μM nicotine did not produce any increase in mIPSC frequency. Nicotine still produced increases in the presence of 5 μM DhβE (B), 40 nM MLA (C), or 40 nM MLA plus 5 μM DhβE (D). (E) Pooled data from individual cells show the mIPSC frequency before and following the application of 100 μM nicotine in the presence of 5 μM Mec (first panel, $n = 8$), 5 μM DhβE (second panel, $n = 12$), 40 nM MLA (third panel, $n = 11$), or 40 nM MLA plus 5 μM DhβE (fourth panel, $n = 6$). (F) The graph shows the changes of mIPSC frequency as percentage of controls following the application of 100 μM nicotine in the presence of 5 μM Mec, 5 μM DhβE, 40 μM MLA, or 40 nM MLA plus 5 μM DhβE.

peak amplitude of nicotine-evoked currents in the presence of DhβE was 27 ± 7 pA (Fig. 8I), and the current lasted for 188 ± 37 s. The mean peak amplitude of nicotine-evoked currents in the presence of DhβE was not significantly different from the currents evoked by nicotine alone ($P = 0.29$). In the third group of cells in the presence of 40 nM MLA, 100 μM nicotine also could elicit inward currents in 12 out of 15 cells tested (Fig. 8H). The mean peak amplitude of nicotine-evoked currents in the presence of MLA was 51 ± 23 pA (Fig. 8I), and the current lasted for 224 ± 32 s. The mean peak amplitude of nicotine-evoked currents in the presence of MLA was also not significantly different from the currents evoked by nicotine alone ($P = 0.23$). Fig. 8I summarizes the profiles of nicotinic agonists and antagonists in the whole-cell current experiments.

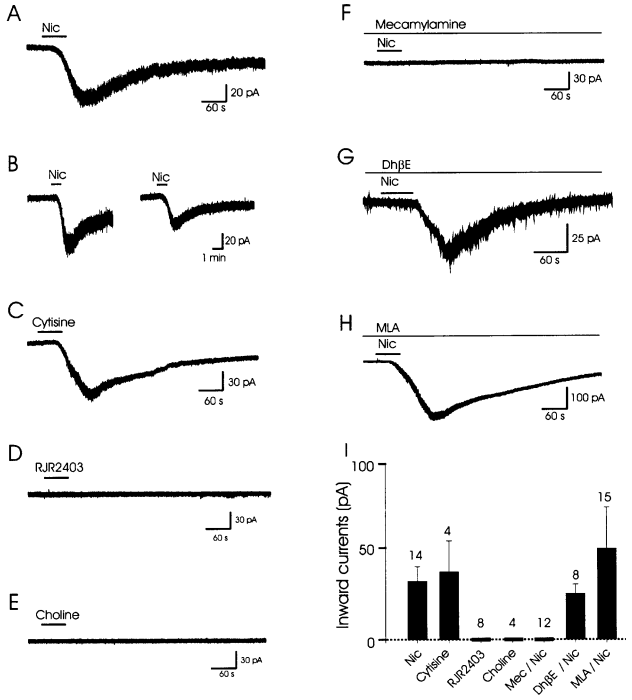

Fig. 8. Whole-cell current directly evoked by nicotine and profiles of nicotinic agonists and antagonists. (A) An example shows a whole-cell current evoked by nicotine. Of 14 cells tested, 13 cells had whole-cell currents following bath application of 100 μM nicotine. (B) Whole-cell currents evoked by 2 nicotine (100 μM) applications. The second application was performed after 60 min wash in normal bath solution. The second nicotinic responses recovered incompletely (37 ± 3% of first responses, $n = 4$, 30–60 min wash). (C–E) Profiles of other nicotinic agonists. (C) An example shows whole-cell current evoked by bath application of 20 μM cytisine. Of four cells tested, all of them showed cytisine-evoked whole-cell currents. (D) RJR-2403 (100 μM) did not evoke any detectable whole-cell current. Of eight cells tested, none of them showed responses. (E) Choline (10 mM) did not produce any detectable whole-cell currents. Of four tested, none of them showed responses. (F–H) Profiles of nicotinic antagonists on nicotine-evoked whole-cell currents. (F) No detectable inward current was evoked by nicotine (100 μM) in the presence of 5 μM mecamylamine in all 12 cells tested. (G) Nicotine (100 μM) still evoked whole-cell currents in the presence of 5 μM DhβE. Of eight cells tested, all of them showed responses to nicotine. (H) Nicotine (100 μM) produced whole-cell currents in the presence of 40 nM MLA. Of 15 cells tested, 12 of them responded and three did not. (I) A summary of the profiles of nicotinic agonists and antagonists. The short solid lines in the graph indicate no inward current. The number above each bar or solid line represents the total number of cells recorded in each experimental condition. All experiments were performed in the presence of 20 μM CNQX, 50 μM APV, 20 μM bicuculline, and 2 μM strychnine. Data represent mean ± SEM.

4. Discussion

We have provided electrophysiological evidence that inhibitory presynaptic terminals in SG express nAChRs and that activation of these receptors produced a robust increase in the release of the inhibitory neurotransmitters GABA and glycine. The agonist and antagonist profiles of synaptic nicotinic responses shown in this study strongly suggest that a subtype of nAChRs, other than the major CNS nAChRs (α7 and α4β2* subtypes), are highly expressed at inhibitory presynaptic terminals in SG region of the spinal cord in adult rats. The enhancement of inhibitory transmission by the activation of these presynaptic nAChRs was widely observed in over 90% cells recorded in SG neurons, suggesting that these presynaptic nAChRs may play an essential role in the inhibitory controls of nociceptive transmission in the SG of the spinal cord.

4.1. Presynaptic localizations of nAChRs in SG

Studies concerning the mechanisms by which nAChRs modulate spontaneous neurotransmitter release have suggested two action sites of nicotine and its analogs. One site is at preterminals, the axonal segments that are close to but not right at the presynaptic sites (Lena et al., 1993; McMahon et al., 1994a). At these sites, nAChR activation produces local depolarization to trigger action potentials, which subsequently result in the release of neurotransmitters. In this case, inhibition of Na^+ channels with TTX can block nAChR-mediated neurotransmitter release. Another potential site of action is at the presynaptic terminals. Alpha7 and α4β2* subtypes of nAChRs have been found to be expressed at presynaptic sites of some CNS neurons. Activation of these presynaptic nAChRs can enhance the release of GABA, glutamate, or other neurotansmitters (McMahon et al., 1994b; McGehee et al., 1995; Gray et al., 1996; Albuquerque et al., 1997; Wonnacott, 1997; Alkondon et al., 1997, 1999; Guo et al., 1998; Li et al., 1998; Mansvelder and McGehee, 1999; Radcliffe et al., 1999; Barazangi and Role, 2001). Unlike preterminal nAChRs, presynaptic nAChR-mediated responses are resistant to TTX (McMahon et al., 1994b, Lena and Changeux, 1997). Our results showed that mIPSC frequency could be enhanced in the presence of TTX, suggesting a high expression of nAChRs at the presynaptic sites of SG inhibitory terminals. Nicotinic AChR activation has been shown to result in the elevation of intracellular Ca^{2+} concentrations. This effect could be mediated by the activation of voltage-gated Ca^{2+} channels due to local depolarization (Noronha-

Blob et al., 1989; Vijayaraghavan et al., 1992; Rathouz and Berg, 1994; Sorimachi, 1995, Lena and Changeux, 1997) or by a direct influx of Ca^{2+} through nAChRs (Mulle et al., 1992; Trouslard et al., 1993; Rathouz and Berg, 1994; Vernino et al., 1994; Rogers and Dani, 1995; Lena and Changeux, 1997). In this study, we have used La^{3+} as a blocker of voltage-gated Ca^{2+} channels (Gu and MacDermott, 1997) to evaluate whether Ca^{2+} entry through presynaptic nAChRs contributes to the increase of GABA/glycine release. In the presence of 30 μM La^{3+}, nicotine still produced increased mIPSC frequency, suggesting that Ca^{2+} entry through SG presynaptic nAChRs directly contributes to the increase of GABA/glycine release.

4.2. Nictonic agonist and antagonist profiles and potential subtypes of nAChRs at SG inhibitory terminals of adult rats

Activation of nAChRs has been shown to modulate the release of a number of neurotransmitters including glutamate, GABA, and monoamines in the brain (Dani, 2001). Agonist and antagonist profiles of nAChR-mediated responses have provided insights into the potential subtypes of nAChRs in different brain regions. For example, many neurons in hippocampus, olfactory bulb, and cerebral cortex were found to highly express α7 nAChRs (Gray et al., 1996; Albuquerque et al., 1997; Girod et al., 2000; Alkondon et al., 2000) and are sensitive to the α7 nAChR-selective agonist choline (Papke et al., 1996; Albuquerque et al., 1997). Furthermore, nicotinic agonist-mediated responses in these regions were sensitive to the blockade by the α7 nAChR selective antagonists MLA and α-bungarotoxin (Alkondon et al., 1997; Gray et al., 1996; Albuquerque et al., 1997; Radcliffe et al., 1999; McGehee, 1999). Another prominent type of nAChR in the CNS region is α4β2*. Alpha4beta2* nAChRs have been found to be highly expressed in many brain regions including ventral lateral geniculate nucleus (Guo et al., 1998), thalamus (Lena and Changeux, 1997) and inhibitory neurons of cerebral cortex (Alkondon et al., 2000). In these regions, nicotinic agonist-mediated responses could be blocked by the α4β2* nAChR selective antagonist DhβE (Alkondon et al., 2000).

We found that cytisine, a relatively selective agonist for α3β4* nAChR receptors (but see Papke and Heinemann, 1994), had similar effects to nicotine in increasing mIPSC frequency. Further, in the presence of both α7 nAChR selective antagonist MLA and the α4β2 receptor-selective antagonist DhβE (Albuquerque et al., 1997; McGehee, 1999), nicotine could still produce a large increase in mIPSC frequency. Our nicotinic agonist and antagonist profiles for the whole-cell currents were also consistent with the profiles of synaptic responses. These results suggest the presence of non-α4β2*, non-α7 nAChR subtype(s) at inhibitory presynaptic terminals in SG of adult rats. Consistently, recent studies with α4 and β2 KO mice also implied the presence of a type(s) of nAChRs in the spinal cord that is different from the major CNS nAChRs (Marubio et al., 1999; Cordero-Erausquin and Changeux, 2001). Evidence has accumulated that some CNS regions may express other subtypes of nAChRs in addition to α4β2* or α7 receptors. For example, it has been shown that medial habenula expressed α3β4 nAChRs, a ganglionic type of nAChRs (Mulle et al., 1991; Quick et al., 1999). Our combined results raised a good possibility that α3β4* nAChRs or a new subtype of nAChRs may be highly expressed at SG inhibitory terminals. A test with the α3β4* nAChRs selective antagonist alpha-conotoxin AuIB should provide clearer answer to this issue (Luo et al., 1998) when this selective antagonist becomes available to research community.

In postnatal rats, nicotinic agonists were shown to facilitate glycine release in the spinal cord DH neurons, which could be completely blocked by 0.3 μM DhβE (Kiyosawa et al. (2001). This study suggested the presence of α4β2* subtype of nAChRs in glycinergic presynaptic terminals of postnatal rats. Another study also showed the presence of DhβE-sensitive nAChRs in DH of postnatal rats (Urban et al., 1989). In contrast, nicotine still produced large increases of sEPSCs and mIPSCs in the presence of 5 μM DhβE in our study with adult rats. There is a possibility that the inhibitory presynaptic terminals in SG of adult rats expressed both α4β2* subtype and a non-α4β2*, non-α7 subtype of nAChRs so that the nicotinic agonist-induced effects could not be abolished in the presence of 5 μM DhβE. Alternatively, α4β2* subtype might be much less common in the SG of adult rats than in the postnatal rats. Consistent with this idea, RJR-2403, a relatively selective agonist to α4β2 nAChR shown in our previous study (Papke et al., 2000), have failed to produce any significant increase in the mIPSC frequency in seven SG neurons and also failed to induce whole-cell currents in eight SG neurons. This raises an interesting possibility that there might be developmental changes in the expression levels of nAChR subtypes in the SG of the spinal cord. In fact, developmental changes of nAChR subunit mRNAs have been observed (Zoli et al., 1995).

We found that nicotine-induced increases of mIPSC frequency were not abolished in the presence of MLA, which is consistent with a recent study in postnatal rats (Kiyosawa et al., 2001). This, however, do not allow us to exclude the possible presence of α7 nAChRs at inhibitory presynaptic terminals of our adult rats. One possibility is that α7 nAChR-mediated responses were masked by the large responses mediated by the MLA- and DhβE-insensitive nAChRs. Another possibility is that the involvement of α7 nAChRs might not be revealed due to the slow penetration of agonists to the tissues in the spinal slices, which could result in desensitization/inactivation of α7 nAChRs. The α7-containing nAChRs are known to desensitize rapidly, making it difficult to observe their actions (Couturier et al., 1990; Gerzanich et al., 1994). The lack of the effects by the selective α7 nAChR agonist choline (Papke et al., 1996) could also be due to the above reason or the presence of strychnine (Matsubayashi et al., 1998). These negative results, on the other hand, strengthen our results

that the observed synaptic effects under our experimental conditions are mediated by non-α7 nAChRs.

In conclusion, modulation of inhibitory synaptic transmission by the non-α4β2, non-α7 nAChRs in the spinal cord DH shown in this study may play a physiological role in nociceptive processing. Our finding provides a new insight into the complicated actions of nicotinic agonists in sensory behaviors observed in different animal models of pain. The molecular identification, i.e. whether they are α3β4*or a new nAChR subtype, remains to be determined.

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