Acetylcholine and Norepinephrine Mediate GABAergic but not Glycinergic Transmission Enhancement by Melittin in Adult Rat Substantia Gelatinosa Neurons

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Running head: GABAergic transmission enhancement by melittin

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Liu T, Fujita T, Kumamoto E. GABAergic and glycineric inhibitory synaptic transmissions in substantia gelatinosa (SG; lamina II of Rexed) neurons of the spinal dorsal horn play an important role in regulating nociceptive transmission from the periphery. It has not yet been well-known whether each of the inhibitory transmissions plays a distinct role in the regulation. We report an involvement of neurotransmitters in GABAergic but not glycineric transmission enhancement produced by phospholipase A2 (PLA₂) activator melittin, where the whole-cell patch-clamp technique is applied to the SG neurons of adult rat spinal cord slices. Glycineric but not GABAergic spontaneous inhibitory postsynaptic current (sIPSC) was increased in frequency and amplitude by melittin in the presence of nicotinic, muscarinic acetylcholine and α₁-adrenergic receptor antagonists (mecamylamine, atropine and WB-4101, respectively). GABAergic transmission enhancement produced by melittin was unaffected by 5-HT₃-receptor and P2X-receptor antagonists (ICS-205,930 and PPADS, respectively). Nicotinic and muscarinic acetylcholine-receptor agonists ((-)nicotine and carbamoylcholine, respectively) and norepinephrine as well as melittin increased GABAergic sIPSC frequency and amplitude. A repeated application of (-)-nicotine, carbamoylcholine and norepinephrine but not melittin at an interval of 30 min produced a similar transmission enhancement. These results indicate that melittin produces the release of acetylcholine and norepinephrine which activate (nicotinic and muscarinic) acetylcholine and α₁-adrenergic receptors, respectively, resulting in GABAergic but not glycineric transmission enhancement in SG neurons. The desensitization of a system leading to the acetylcholine and norepinephrine release is slow in recovery. This distinction in modulation between GABAergic and glycineric transmissions may play a role in regulating nociceptive transmission.
INTRODUCTION

A neuronal circuitry in the superficial dorsal horn, particularly the substantia gelatinosa (SG, lamina II of Rexed), plays an important role in modulating nociceptive transmission to the CNS from the periphery through glutamatergic primary-afferent fibers (for review see Mason 1999; Melzack and Wall 1965; Willis and Coggeshall 1991). Glutamatergic excitatory synaptic transmission in SG neurons is inhibited by a variety of endogenous and exogenous analgesics including opioids (Fujita and Kumamoto 2006; Kohno et al. 1999; Wu et al. 2003), baclofen (Blake et al. 1993; Iyadomi et al. 2000), nociceptin (Liebel et al. 1997; Luo et al. 2002) and adenosine (Lao et al. 2004; Li and Perl 1994; for review see Fürst 1999). GABAergic and glycinergic inhibitory interneurons in the SG are involved in polysynaptic pathways originating in primary-afferent terminals (Coggeshall and Carlton 1997; Todd et al. 1996; Willis and Coggeshall 1991), and possibly serve to modulate nociceptive transmission. In support of this idea, the lack of GABA-synthesizing enzyme (Kohno 2007; Moore et al. 2002) and also reduction in the expression of K⁺-Cl⁻ exporter KCC2, which causes inhibitory synaptic response to be excitatory (Coull et al. 2003), in the rat spinal dorsal horn lead to nociception. Peripheral inflammation results in a reduced glycinergic transmission in rat spinal lamina I neurons by a presynaptic mechanism (Müller et al. 2003). An increase in endogenous glycine due to glycine transporter-1 blockade produces an inhibitory effect on spinal nociceptive transmission (Tanabe et al. 2008; for review see Sandkühler 2009).

Phospholipase A₂ (PLA₂) plays an important role in nociception through the synthesis of eicosanoids by the actions of cyclooxygenase (COX; for review see Samad et al. 2002; Svensson and Yaksh 2002; Vanegas and Schaible 2001) and lipoxygenase (LOX; for instance see Hwang et al. 2000) in the spinal dorsal horn (for review see Dennis 1994). There is much evidence supporting this idea. For example, the rat
spinal cord contains PLA2 (Samad et al. 2001); peripheral tissue injury and inflammation increase the release of prostaglandin E2 (PGE2; one of COX metabolites) and produce PLA2-mediated glutamatergic excitatory transmission enhancement in the spinal dorsal horn (Dirig and Yaksh 1999; Young et al. 1995). Bath-applied PGE2 produces a membrane depolarization in the SG neurons of rat spinal cord slices (Baba et al. 2001). PGE2 inhibits glycinergic inhibitory transmission in rat superficial dorsal horn neurons (Ahmadi et al. 2002; for review see Zeilhofer 2005). Hwang et al. (2000) have demonstrated that the metabolites of LOX directly activate transient receptor potential type-1 channel that plays a role in regulating nociceptive transmission in the SG (Jiang et al. 2009; for review see Szallasi 2002).

We have recently revealed by using a 26 amino-acid peptide melittin that PLA2 activation enhances GABAergic and glycinergic spontaneous inhibitory synaptic transmissions in all of the rat SG neurons examined (Liu et al. 2008). This is so, although the SG is composed of a heterogeneous cell group (Grudt and Perl 2002). The glycinergic transmission enhancement was due to PLA2 and subsequent LOX activation at glycinergic synapses while the GABAergic transmission one was suggested to be mediated by neurotransmitters released from neurons as a result of excitatory transmission enhancement caused by PLA2 activation (see Yue et al. 2005) and subsequent increase in neuronal activities (Liu et al. 2008). There are a variety of neurotransmitters such as acetylcholine, norepinephrine, ATP and serotonin which are involved in enhancing spontaneous inhibitory transmission in spinal dorsal horn neurons (Abe et al. 2009; Baba et al. 1998, 2000a,b; Fukushima et al. 2009; Kawamata et al. 2003; Kiyosawa et al. 2001; Rhee et al. 2000; Takeda et al. 2003). The present study examined what kinds of neurotransmitters are involved in the melittin-induced GABAergic spontaneous inhibitory transmission enhancement by applying the whole-cell patch-clamp technique to the SG neurons of adult rat spinal cord slice preparations.
METHODS

This study was approved by the Animal Care and Use Committee of Saga University, and was conducted in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Science of the Physiological Society of Japan. All efforts were made to minimize animal suffering and the number of animals used.

Slice preparation

Spinal cord slices from adult rats were prepared as described previously (Fujita and Kumamoto 2006; Jiang et al. 2009; Liu et al. 2004, 2008). In brief, male adult Sprague-Dawley rats (6-8 weeks old) were anesthetized with urethane (1.2 g/kg, intraperitoneal), and then a laminectomy was performed to extract a lumbosacral spinal cord enlargement (L1-S3). The spinal cord was 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 and then a 600-μm thick transverse slice was cut. The slice was placed on a nylon mesh in the recording chamber (volume: 0.5 ml), and then perfused at a rate of 10-15 ml/min with Krebs solution bubbled with 95 % O₂ and 5 % CO₂, and maintained at 36 ± 1 °C. The Krebs solution contained NaCl, 117; KCl, 3.6; CaCl₂, 2.5; MgCl₂, 1.2; NaH₂PO₄, 1.2; NaHCO₃, 25; and glucose, 11 (in mM; pH = 7.4 when saturated with the gas).

Whole-cell voltage-clamp recordings

The SG was identified as a translucent band under a binocular microscope with
light transmitted from below (Fujita and Kumamoto 2006; Jiang et al. 2009; Liu et al. 2004, 2008). Blind whole-cell voltage-clamp recordings of spontaneous inhibitory postsynaptic currents (sIPSCs) from the SG neurons were made at a holding potential \( V_{\text{H}} \) of 0 mV where glutamatergic spontaneous excitatory postsynaptic currents (EPSCs) were invisible because the reversal potential for non-NMDA receptor channels involved in this production was close to 0 mV (Iyadomi et al. 2000; Kohno et al. 1999; Liu et al. 2008; Luo et al. 2002; Yang et al. 2004). Patch-pipettes were fabricated from thin-walled, fiber-filled capillaries (1.5 mm o.d.) and contained the following solution (in mM): \( \text{Cs}_2\text{SO}_4, 110; \text{CaCl}_2, 0.5; \text{MgCl}_2, 2; \text{EGTA}, 5; \text{HEPES}, 5; \text{Mg-ATP}, 5; \) and tetraethylammonium (TEA)-Cl, 5 (pH = 7.2). \( \text{Cs}^+ \) and TEA were added to inhibit \( \text{K}^+ \) channels located in the recorded SG neurons and thus to easily shift \( V_{\text{H}} \) to 0 mV from resting membrane potentials. Signals were acquired using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Currents obtained in the voltage-clamp mode were low-pass-filtered at 5 kHz, and digitized at 333 kHz with an A/D converter (Digidata 1322A, Molecular Devices). The data were stored and analyzed with a personal computer using pCLAMP v 9.2 software (Molecular Devices). The program (AxoGraph 4.0, Molecular Devices) used for analyzing sIPSCs detects spontaneous events if the difference between the baseline and a following current value exceeds a given threshold of 5 pA and separating valleys are less than 50 % of adjacent peaks. Width at a half of the peak of sIPSC was also analyzed by using AxoGraph 4.0.

**Application of drugs**

Drugs were applied by perfusing a solution containing drugs of a known concentration without an alteration in the perfusion rate and temperature. The solution in the recording chamber having a volume of 0.5 ml was completely replaced within 15 s. The drugs used were bicuculline methiodide, tetrodotoxin (TTX; Wako, Osaka,
Japan), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), DL-2-amino-5-phosphonovaleric acid (APV; Tocris Cookson, Bristol, UK), atropine sulfate (Tokyo Kasei, Japan), melittin purified from bee venom, 4-bromophenacyl bromide (4-BPB), strychnine nitrate, mecamylamine hydrochloride, (-)-nicotine, carbamoylcholine, norepinephrine, WB-4101, 3-tropanylindole-3-carboxylate (ICS-205930) methiodide and pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS; Sigma, St. Louis, MO). These drugs (except for CNQX where dimethyl sulfoxide was used as solvent) were first dissolved in distilled water at 1000 times the concentration to be used and then stored at -20 °C. The stock solution was diluted to the desired concentration in Krebs solution immediately before use. When melittin (1 μM) was repeatedly superfused in the same spinal cord slice, time intervals between the applications were more than 1 h, unless otherwise mentioned.

Statistical analysis

Numerical data are presented as the mean ± SE. Statistical significance was determined as \( P < 0.05 \) using paired (unless otherwise mentioned), unpaired Student’s \( t \) test, or ANOVA followed by Turkey-Kramer’s post hoc test. In all cases \( n \) refers to the number of neurons studied.

RESULTS

SG neurons had resting membrane potentials more negative than -60 mV when measured in a current-clamp mode just after the establishment of whole-cell mode. When \( V_H \) was shifted to 0 mV in a voltage-clamp mode, sIPSCs were observed in all SG neurons examined. GABAergic and glycineergic sIPSCs were recorded in the presence of a glycine-receptor antagonist strychnine (1 μM) and a GABA\(_A\)-receptor
The effects of a variety of receptor antagonists on spontaneous inhibitory transmission enhancement produced by melittin in SG neurons

Since the activation of nicotinic and muscarinic acetylcholine receptors enhanced spontaneous inhibitory transmission in adult rat SG neurons (Baba et al. 1998; Kiyosawa et al. 2001; Takeda et al. 2003), we first examined how GABAergic and glycinergic spontaneous inhibitory transmission enhancements produced by melittin (1 μM) superfused for 3 min are affected by a nicotinic acetylcholine-receptor antagonist mecamylamine (20 μM) or a muscarinic acetylcholine-receptor antagonist atropine (1 μM). In the presence of mecamylamine or atropine, melittin did not significantly enhance GABAergic synaptic transmission albeit there was a tendency to be enhanced (data not shown). GABAergic sIPSC frequency and amplitude around 3 min after the beginning of melittin superfusion in the presence of mecamylamine were, respectively, 184 ± 75 % (n = 6; P > 0.05) and 132 ± 36 % (n = 6; P > 0.05) of control (2.6 ± 0.6 Hz and 11.5 ± 1.0 pA); those in the presence of atropine were, respectively, 132 ± 17 % (n = 5; P > 0.05) and 140 ± 51 % (n = 5; P > 0.05) of control (2.7 ± 0.8 Hz and 12.0 ± 0.7 pA). These GABAergic sIPSC frequency and amplitude percentages were significantly smaller than those (frequency and amplitude: 386 ± 37 % and 178 ± 10 %, respectively; n = 32; see Liu et al. 2008) produced by melittin (1 μM) in the absence of these acetylcholine-receptor antagonists (P < 0.05). In the presence of both mecamylamine and atropine, melittin did not affect GABAergic inhibitory transmission, as seen in Fig. 1Aa. Figure 1Ab summarizes data about GABAergic sIPSC frequency and amplitude, as obtained from seven neurons. On the contrary, glycinergic
inhibitory transmission was enhanced by melittin (1 μM) in the presence of both mecamylamine and atropine, as seen in Fig. 1Ba. Glycinergic sIPSC frequency and amplitude percentages, relative to control, around 3 min after the beginning of melittin superfusion (293 ± 34 % and 166 ± 17 %, respectively; n = 7) were significantly not different from those (frequency and amplitude: 391 ± 36 % and 207 ± 13 %, respectively; n = 27; see Liu et al. 2008) produced by melittin (1 μM) in the absence of the acetylcholine-receptor antagonists (P > 0.05), as summarized in Fig. 1Bb.

Since norepinephrine enhanced spontaneous inhibitory transmission in SG neurons by activating α1 adrenoceptors (Baba et al. 2000a,b), we next examined how an α1-adrenoceptor antagonist WB-4101 (0.5 μM) affects GABAergic and glycinergic spontaneous inhibitory transmission enhancements produced by melittin (1 μM). Melittin enhanced glycinergic but not GABAergic inhibitory transmission in the presence of WB-4101, as seen in Figs. 2Aa, 2Ba. Similar actions of melittin on GABAergic and glycinergic inhibitory transmissions were obtained from other four and five neurons, respectively; these results were summarized in Figs. 2Ab, 2Bb.

No effect of acetylcholine-receptor and α1-adrenoceptor antagonists on glycinergic spontaneous inhibitory transmission enhancement produced by melittin suggests a direct action of melittin on glycinergic transmission together with the observations reported previously that a Na+-channel blocker TTX, glutamate-receptor antagonists (non-NMDA and NMDA receptor-antagonists: CNQX and APV, respectively) and nominally Ca2+-free solution do not affect melittin-induced glycinergic transmission enhancement (Liu et al. 2008). On the contrary, the inhibition of GABAergic spontaneous inhibitory transmission enhancement produced by melittin in the presence of acetylcholine-receptor or α1-adrenoceptor antagonists suggests an involvement of acetylcholine and/or norepinephrine. 5-HT3-receptor activation produced an increase in GABA release in the rat spinal dorsal horn (Abe et al. 2009; Fukushima et al. 2009; Kawamata et al. 2003) and ATP enhanced spontaneous
inhibitory transmission in rat SG neurons by activating P2X receptors (Rhee et al. 2000). Therefore, we further examined whether a 5-HT$_3$-receptor antagonist ICS-205,930 (2 nM, a concentration enough to inhibit presynaptic 5-HT$_3$ receptors involved in facilitating GABA release in the rat hippocampus; Turner et al. 2004) or P2X-receptor antagonist PPADS (10 μM, a concentration enough to inhibit P2X-receptor mediated enhancement of glycine release from nerve terminals in the spinal dorsal horn; Rhee et al. 2000) affects GABAergic spontaneous inhibitory transmission enhancement produced by melittin (1 μM). As seen in Figs. 3A, 3B, melittin enhanced GABAergic inhibitory transmission in the presence of ICS-205,930 or PPADS. As summarized in Fig. 3C, melittin-induced GABAergic sIPSC frequency and amplitude increases in the presence of ICS-205,930 or PPADS were not significantly different from those in the absence of the antagonists ($P > 0.05$).

GABAergic spontaneous inhibitory transmission was not enhanced by melittin (1 μM) under the pretreatment for 4 min with a PLA$_2$ inhibitor 4-BPB (10 μM; Mayer and Marshall 1993), as seen for glycinergic spontaneous transmission (Liu et al. 2008); the frequency and amplitude around 3 min after the beginning of melittin superfusion were 137 ± 18 % ($n = 9$; $P > 0.05$) and 95 ± 5 % ($n = 9$; $P > 0.05$), respectively, of control (1.9 ± 0.3 Hz and 10.9 ± 0.6 pA). This result indicates that the melittin-induced GABAergic transmission enhancement is also mediated by PLA$_2$, although there is a difference in the sensitivity to cholinergic and α$_1$-adrenergic receptor antagonists between GABAergic and glycinergic transmission enhancements produced by melittin.

The effects of melittin, nicotinic, muscarinic acetylcholine-receptor agonists and norepinephrine on GABAergic spontaneous inhibitory transmission in SG neurons

Since GABAergic spontaneous transmission in SG neurons was reported to be
enhanced by acetylcholine-receptor and α₁-adrenoceptor agonists (Baba et al. 1998, 2000a,b; Takeda et al. 2003), we examined the effect of a nicotinic, muscarinic acetylcholine-receptor agonist, [(-)-nicotine (50 μM) and carbamoylcholine (10 μM), respectively] or norepinephrine (20 μM) on GABAergic spontaneous inhibitory transmission in neurons where the effect of melittin (1 μM) on the inhibitory transmission was examined. These results are given in Figs. 4-6. About 30 min after melittin superfusion, either (-)-nicotine, carbamoylcholine or norepinephrine as well as melittin facilitated GABAergic inhibitory transmission (see A,B in Figs. 4-6), as reported previously (Baba et al. 1998, 2000a,b; Takeda et al. 2003). Similar actions of (-)-nicotine, carbamoylcholine and norepinephrine together with melittin on GABAergic transmission were obtained from other 8, 9 and 15 neurons, respectively; these results were summarized in Figs. 4C-6C.

Since a repeated application of melittin (1 μM) did not enhance glycinergic spontaneous inhibitory transmission (Liu et al. 2008), we next examined whether this is so for the melittin-induced enhancement of GABAergic spontaneous inhibitory transmission. Figure 7 demonstrates the effect of a repeated application of melittin (1 μM) on GABAergic transmission in the same neuron. When melittin was once again applied 30 min after its washout, the effect of the second application of melittin on GABAergic transmission (Fig. 7B) was much smaller than that of the first application of melittin (Fig. 7A). Figure 7C shows a comparison of relative frequency and amplitude of GABAergic sIPSC under the action of melittin to those before its superfusion between the first and second applications, which is obtained from 6 neurons.

The second application of melittin did not largely affect GABAergic spontaneous inhibitory transmission at 30 min after its first application (Fig. 7) while the transmission was enhanced by (-)-nicotine, carbamoylcholine or norepinephrine around 30 min after the first application of melittin (Figs. 4-6). These results suggest that a process leading to the release of acetylcholine and/or norepinephrine as a result of
melittin action may be slow in recovery. In order to confirm this idea, we next examined how a repeated application of (-)-nicotine (50 μM), carbamoylcholine (10 μM) or norepinephrine (20 μM) affects GABAergic spontaneous inhibitory transmission. As seen from Figs. 8A-8C, each of them repeatedly facilitated GABAergic transmission. The initial application of (-)-nicotine increased GABAergic sIPSC frequency and amplitude [320 ± 43 % (n = 8; P < 0.05) and 151 ± 12 % (n = 8; P < 0.05), respectively, of control (2.6 ± 0.5 Hz and 10.2 ± 1.0 pA) around 1 min after the beginning of its superfusion]. A second application 30 min later increased GABAergic sIPSC frequency and amplitude [288 ± 35 % (n = 8; P < 0.05) and 128 ± 6 % (n = 8; P < 0.05), respectively, of control (2.6 ± 0.5 Hz and 10.0 ± 1.0 pA)]; these percentage values were not significantly different from those of the initial application (P > 0.05).

Similar results were obtained for carbamoylcholine and norepinephrine actions. The initial application of carbamoylcholine increased GABAergic sIPSC frequency and amplitude [321 ± 49 % (n = 4; P < 0.05) and 236 ± 37 % (n = 4; P < 0.05), respectively, of control (3.1 ± 0.3 Hz and 10.0 ± 0.9 pA) around 1 min after the beginning of its superfusion]. A second application 30 min later did so with a similar extent; sIPSC frequency and amplitude: 241 ± 12 % (n = 4; P < 0.05) and 206 ± 27 % (n = 4; P < 0.05), respectively, of control (4.1 ± 0.3 Hz and 9.9 ± 0.6 pA). The initial application of norepinephrine increased GABAergic sIPSC frequency and amplitude [470 ± 18 % (n = 3; P < 0.05) and 342 ± 40 % (n = 3; P < 0.05), respectively, of control (1.7 ± 0.4 Hz and 9.2 ± 0.5 pA) around 1 min after the beginning of its superfusion]. A second application 30 min later enhanced GABAergic transmission with a similar extent; sIPSC frequency and amplitude: 363 ± 25 % (n = 3; P < 0.05) and 332 ± 34 % (n = 3; P < 0.05), respectively, of control (2.3 ± 0.5 Hz and 10.3 ± 0.9 pA).

Difference in melittin effect between GABAergic and glycinergic spontaneous inhibitory transmissions in the same neuron
The difference in melittin-induced enhancement in the presence of acetylcholine-receptor or \( \alpha_1 \)-adrenoceptor antagonist between GABAergic and glycinergic spontaneous inhibitory transmissions was examined in Krebs solution containing strychnine or bicuculline (see Figs. 1 and 2). In order to know whether this difference is seen in Krebs solution without strychnine and bicuculline, we analyzed the half-width time of sIPSC in the absence and absence of melittin (1 \( \mu \)M), recorded in the presence of strychnine or bicuculline and in the absence of them. Consistent with the previous observation that the half-decay time of GABAergic sIPSC is about three-fold larger than that of glycinergic sIPSC (Kohno et al. 1999; Luo et al. 2002; Yang et al. 2004), the distribution of the half-width time of GABAergic sIPSC shifted to a larger one than that of glycinergic sIPSC, as noted from Fig. 9A. In each of GABAergic and glycinergic transmissions, the numbers of events having individual half-width times of GABAergic or glycinergic sIPSC increased under the action of melittin (see Fig. 9A).

Similar results for GABAergic and glycinergic transmissions were obtained from other 5 and 5 neurons, respectively. Averaged half-width times of GABAergic sIPSC in the control and under the action of melittin were 9.2 ± 0.6 ms (\( n = 6 \)) and 10.7 ± 0.9 ms (\( n = 6 \)), respectively; melittin increased a total of its event numbers by 320 ± 70 % (\( n = 6; P < 0.05 \)). Averaged half-width times of glycinergic sIPSC in the control and under the action of melittin were 2.9 ± 0.2 ms (\( n = 6 \)) and 3.8 ± 0.3 ms (\( n = 6 \)), respectively; melittin increased a total of its event numbers by 520 ± 160 % (\( n = 6; P < 0.05 \)). The half-width time of glycinergic sIPSC was significantly smaller than that of GABAergic sIPSC (\( P < 0.05 \); unpaired t test). Figure 9Ba demonstrates the effect of melittin (1 \( \mu \)M) on inhibitory transmission recorded in the presence of TTX (0.5 \( \mu \)M), CNQX (10 \( \mu \)M) and APV (50 \( \mu \)M) where melittin is expected to enhance glycinergic transmission without any change in GABAergic transmission (see Liu et al. 2008). As noted from Fig. 9Bb, consistent with this expectation, melittin increased the number of events
having shorter half-width time corresponding to that of glycinergic sIPSC (see Fig. 9Ab) whereas unchanging one having longer half-width time which was comparable to that of GABAergic sIPSC (see Fig. 9Aa). Similar results were obtained from other 3 neurons. The peak values of the half-width time of sIPSC under the action of melittin averaged to be $2.8 \pm 0.3$ ms ($n = 4$), values comparable to those of glycinergic sIPSC (2.9-3.8 ms) but not GABAergic sIPSC (9.2-10.7 ms) as given above.

There was a difference in melittin effect between GABAergic and glycinergic spontaneous inhibitory transmissions, whereas Jonas et al. (1998) reported that GABA and glycine are co-released from a single nerve terminal in the neonate rat ventral horn. If such a co-release occurs in the adult rat SG, it is unlikely that GABAergic and glycinergic transmissions are affected by melittin in a manner different from each other. In order to test the possibility that GABA and glycine are co-released from SG nerve terminals in rats younger than those used in the present study, we examined how melittin (1 μM) affects each of the inhibitory transmissions in SG neurons of young rats (2-3 weeks old) in the presence of TTX (0.5 μM), CNQX (10 μM) and APV (50 μM). Melittin enhanced GABAergic but not glycinergic transmission under this condition. Around 3 min after the beginning of melittin superfusion, GABAergic sIPSC frequency and amplitude were, respectively, $1629 \pm 875$ % ($n = 3$) and $105 \pm 18$ % ($n = 3$) of control ($1.1 \pm 0.5$ Hz and $8.9 \pm 0.5$ pA). On the contrary, glycinergic sIPSC frequency and amplitude were, respectively, $76 \pm 26$ % ($n = 3$) and $80 \pm 14$ % ($n = 3$) of control ($2.8 \pm 1.0$ Hz and $10.0 \pm 1.6$ pA; unpublished observations by Chung-Yu Jiang). This result suggests that GABA and glycine may not be co-released from SG nerve terminals in young as well as adult rats.

**DISCUSSION**

The present study demonstrated that a PLA$_2$ activator melittin increased the
frequency and amplitude of GABAergic but not glycinerigic sIPSC in adult rat SG neurons in a manner sensitive to nicotinic, muscarinic acetylcholine-receptor and/or α1-adrenoceptor antagonists. Nicotinic and muscarinic acetylcholine-receptor agonists [(-)-nicotine and carbamoylcholine, respectively] and norepinephrine as well as melittin enhanced GABAergic sIPSC frequency and amplitude in SG neurons, suggesting that the GABAergic transmission enhancement produced by melittin was mediated by acetylcholine and norepinephrine released from neurons as a result of an action of melittin. Acetylcholine and norepinephrine so released activated (nicotinic and muscarinic) acetylcholine-receptors and α1 adrenoceptors, respectively. This idea is consistent with our previous observations that the GABAergic but not glycinerigic transmission enhancement produced by melittin was reduced in extent in Na+-channel blocker TTX-, glutamate-receptor antagonists (CNQX and APV)-containing or nominally Ca2+-free Krebs solution (Liu et al. 2008). In support of our idea, the spinal dorsal horn including the SG contains nicotinic, muscarinic acetylcholine-receptor and α1-adrenoceptor subunits (Pieribone et al. 1994; Wada et al. 1989, 1990; Yamamura et al. 1983). The GABAergic transmission enhancements produced by nicotine, carbamoylcholine and norepinephrine have been already reported in adult rat SG neurons (Baba et al. 1998, 2000a,b; Takeda et al. 2003).

Carbamoylcholine and norepinephrine increased GABAergic sIPSC frequency and amplitude in an action potential-dependent and -independent manner (Baba et al. 1998, 2000a) while nicotine enhanced GABAergic sIPSC frequency and amplitude without the production of action potentials (Takeda et al. 2003). These results suggest that the GABAergic sIPSC frequency and amplitude increase produced by melittin are due to an increase in the release of GABA from nerve terminals without and with the production of action potentials in neurons presynaptic to SG neurons as a result of the actions of acetylcholine and norepinephrine. It is unlikely that the GABAergic sIPSC amplitude increase produced by melittin is due to an increase in the affinity of GABA_A...
receptors for GABA, as shown for AMPA receptors (Bernard et al. 1995; Cruickshank and Henley 1994; Yue et al. 2005), because this amplitude increase is not seen in the presence of acetylcholine-receptor or \( \alpha_1 \)-adrenoceptor antagonists.

There are several possible origins of acetylcholine and norepinephrine which may be involved in the melittin action. Immunohistochemical studies of choline acetyltransferase have demonstrated the presence of intrinsic spinal cholinergic neurons in the deep spinal dorsal horn, particularly the lamina III, of adult rats (Borges and Iversen 1986); these neurons terminate in the superficial dorsal horn including the SG (Ribeiro-da-Silva and Cuello 1990). These results are consistent with the observation that a focal stimulation of the deep dorsal horn results in GABAergic spontaneous transmission enhancement similar to that produced by carbamoylcholine in SG neurons (Baba et al. 1998). Anatomical and behavioral studies have revealed the existence of a descending cholinergic pathway to the spinal dorsal horn from the brain stem in the rat (Jones et al. 1986; Zhuo and Gebhart 1990). There is much evidence showing the presence of descending norepinephrine-containing fibers to the spinal dorsal horn, particularly the SG, from the brain stem in rats (Westlund et al. 1983; for review see Jones 1991). Thus, it seems that descending neurons from the brain stem release acetylcholine and norepinephrine in the SG while acetylcholine also originates from spinal intrinsic neurons. Since a repeated application of \((-\)\)-nicotine, carbamoylcholine and norepinephrine enhanced GABAergic transmission while melittin did not so, acetylcholine and norepinephrine release produced by melittin appeared to be slow in recovery after once the release occurred. Such a release of neurotransmitters may be due to an increase in intraterminal \( \text{Ca}^{2+} \) concentrations as a result of melittin actions, because melittin increases intracellular \( \text{Ca}^{2+} \) concentrations in rat hippocampal mossy fiber nerve endings by activating PLA\(_2\) (Damron and Dorman 1993). A desensitization of this intracellular pathway leading to neurotransmitter release may result in an inhibition of the effect of the second application of melittin on spontaneous
GABAergic transmission. It remains to be examined how melittin action, i.e., PLA₂ activation, results in the release of acetylcholine and norepinephrine from the neurons.

The present and our previous observations (Liu et al. 2008) indicate that melittin increases GABAergic and glycinergic sIPSC frequency in a manner different from each other. This difference between GABAergic and glycinergic transmissions was also observed in the absence of both bicuculline and strychnine (see Fig. 9). The glycinergic sIPSC frequency increase produced by melittin was significantly unaffected by acetylcholine-receptor or α₁-adrenoceptor antagonists, suggesting that even if acetylcholine and norepinephrine are released as a result of melittin action, they do not enhance glycinergic sIPSC frequency in spite of their ability to increase the frequency in SG neurons (Baba et al. 2000b; Kiyosawa et al. 2001; Takeda et al. 2003).

Altogether, our results suggest that GABA and glycine may be released from a different nerve terminal containing either GABA or glycine. This result in the SG is different from those in the adult rat lamina I (Chéry and de Koninck 1999) and also in the neonate rat ventral horn (Jonas et al. 1998) where GABA and glycine are thought to be co-released from a single nerve terminal. The difference between our and previous other studies appears not to be due to a distinction in age among the rats used, because the difference in melittin effect between GABAergic and glycinergic transmissions was seen in young rats (2-3 weeks old), where GABAergic but not glycinergic transmission was enhanced by melittin in the presence of TTX, CNQX and APV. This result was reversed to that in adult rats (see Fig. 9Ba). This reversal may be attributed to a developmental change in processes such as melittin-mediated PLA₂ activation leading to neurotransmitter release enhancement. This issue remains to be further examined.

Our idea that GABA and glycine may not be co-released is supported by an immunohistochemical observation that some presynaptic terminals in the SG contain GABA without glycine (Spike and Todd 1992). Such GABAergic terminals may originate from neurons located in laminae deeper than the SG (Todd and Sullivan 1990).
or from descending nerve fibers in the rostral ventromedial medulla (Antal et al. 1996); the latter idea is supported by a direct GABAergic inhibition of the SG from the rostral ventromedial medulla as revealed by in vivo patch-clamp recordings (Kato et al. 2006). Moreover, neuropeptide Y, galanin and enkephalin that are identified in GABAergic interneurons in laminae I-III of the dorsal horn have been reported to be restricted to those neurons that are not glycinergic (Todd and Koerber 2005). Alternatively, Baba et al. (2000b) have demonstrated that a half-maximal effective concentration for norepinephrine in increasing GABAergic sIPSC frequency (29.5 μM) is different from that for glycinergic sIPSC frequency (38.8 μM) in SG neurons.

GABAergic and glycinergic inhibitory transmissions in the superficial dorsal horn are modulated by opioids, nociceptin, nicotine and adenosine in a manner similar to each other. A μ-opioid receptor agonist (DAMGO) and nociceptin did not affect both of GABAergic and glycinergic inhibitory transmissions in SG neurons (Kohno et al. 1999; Luo et al. 2002). Nicotine increased GABAergic and glycinergic sIPSC frequency in SG neurons by almost the same extent (Takeda et al. 2003). Yang et al. (2004) have reported that GABAergic and glycinergic sIPSC frequency in SG neurons are reduced by adenosine with almost the same half-maximal effective concentration. On the other hand, glycinergic but not GABAergic inhibitory transmission in the superficial dorsal horn was inhibited by PGE2; this inhibition was postsynaptic in origin, i.e., a modulation of glycine- but not GABA\(\alpha\)-receptor channels by PGE2 (Ahmadi et al. 2002; Zeilhofer 2005). The present study revealed that GABAergic but not glycinergic sIPSC frequency in SG neurons is augmented by acetylcholine and norepinephrine released as a result of melittin action and thus PLA2 activation. This is, to our knowledge, the first demonstration of a clear-cut difference in presynaptic modulation between GABAergic and glycinergic transmissions in the spinal cord.

Physiological significance of GABAergic inhibitory transmission enhancement
mediated by acetylcholine and norepinephrine

A neuronal circuitry in the SG plays an important role in modulating nociceptive transmission to the CNS from the periphery (Mason 1999; Melzack and Wall 1965; Willis and Coggeshall 1991). PLA₂ activation in the SG may occur following a repeated application of mustard oil to the periphery (Young et al. 1995). Alternatively, PLA₂ in the SG may be activated by a peptide analogous to melittin in the mammal (Clark et al. 1991) or an increase in intracellular Ca²⁺ concentration due to an increase in synaptic activities such as an excess of glutamate-receptor activation (Williams et al. 1989; for review see Meller and Gebhart 1994). There is much evidence showing that acetylcholine and norepinephrine released following PLA₂ activation as suggested in the present study are involved in inhibiting nociceptive transmission in the spinal dorsal horn. When administered intrathecally, acetylcholine and norepinephrine produce antinociception (Abram and O'Connor 1995; Abram and Winne 1995; Howe et al. 1983; Khan et al. 1998, 2001; Reddy et al. 1980). Mice lacking muscarinic acetylcholine-receptor M2-type or nicotinic acetylcholine-receptor α₄ and β₂ subunits display a reduction in antinociception produced by agonists of the receptors, as assessed in the hot plate and tail-flick tests (Gomeza et al. 1999; Marubio et al. 1999). The antinociceptive actions mediated by acetylcholine and norepinephrine have been partly attributed to their facilitatory actions on GABAergic and glycinergic inhibitory transmissions, which are expected to reduce the excitability of SG neurons (Baba et al. 1998, 2000a,b; Takeda et al. 2003). Such a GABAergic inhibitory transmission enhancement as that produced by melittin and thus PLA₂ activation, revealed in the present study, could contribute to antinociception. Considering that such a melittin action is not observed for glycinergic transmission, GABAergic and glycinergic inhibitory transmissions in the SG may play a distinct role in regulating nociceptive transmission.
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DISCLOSURES

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Figure legends

FIG. 1. Glycinergic but not GABAergic spontaneous inhibitory transmission is facilitated by melittin (1 μM) in the presence of nicotinic and muscarinic acetylcholine-receptor antagonists [mecamylamine (20 μM) and atropine (1 μM), respectively]. A, B: effects of melittin on GABAergic (A) and glycinergic inhibitory transmission (B) in the presence of mecamylamine and atropine. Aa, Ba: recordings of sIPSCs in the absence and presence of melittin in Krebs solution containing mecamylamine and atropine. In this and subsequent figures, a bar shown above the recording indicates a period during which drugs are superfused; four traces, which are shown in an expanded scale in time, indicate sIPSCs recorded consecutively for a period indicated by a bar shown below the recording. Ab, Bb: sIPSC frequency (left) and amplitude (right) under the action of melittin (measured around 3 min after the beginning of its superfusion), relative to those before melittin superfusion, in Krebs solution without (Ab: n = 32; Bb: n = 27; these data were taken from Liu et al. 2008) and with mecamylamine and atropine [just before melittin superfusion: 2.0 ± 0.2 Hz and 10.4 ± 0.7 pA (n = 7) in Ab; 2.2 ± 0.5 Hz and 10.2 ± 1.2 pA (n = 7) in Bb]. In this and subsequent figures, vertical lines accompanied by bars indicate SE; statistical significance between data shown by bars is indicated by asterisks, * and **: P < 0.05 and P < 0.001, respectively; n.s.: not significant; comparison between melittin effects in the absence and presence of receptor antagonists is performed by using ANOVA followed by Turkey-Kramer’s post hoc test. GABAergic and glycinergic inhibitory transmissions were recorded in the presence of a glycine-receptor antagonist strychnine (1 μM) and a GABA_A-receptor antagonist bicuculline (10 μM), respectively. V_H = 0 mV.

FIG. 2. Glycinergic but not GABAergic spontaneous inhibitory transmission is
facilitated by melittin (1 μM) in the presence of an α₁-adrenoceptor antagonist WB-4101 (0.5 μM). *A, B*: effects of melittin on GABAergic (*A*) and glycinergic inhibitory transmission (*B*) in the presence of WB-4101. *Aa, Ba*: recordings of sIPSCs in the absence and presence of melittin in Krebs solution containing WB-4101. *Ab, Bb*: sIPSC frequency (*left*) and amplitude (*right*) under the action of melittin (measured around 3 min after the beginning of its superfusion), relative to those before melittin superfusion, in Krebs solution without (*Ab: n = 32; Bb: n = 27; these data were taken from Liu et al. 2008*) and with WB-4101 [just before melittin superfusion: 2.4 ± 0.8 Hz and 12.9 ± 1.3 pA (*n = 5*) in *Ab; 2.0 ± 0.4 Hz and 11.3 ± 0.6 pA (*n = 6*) in *Bb*]. GABAergic and glycinergic inhibitory transmissions were recorded in the presence of strychnine (1 μM) and bicuculline (10 μM), respectively. $V_H = 0$ mV.

**FIG. 3.** GABAergic spontaneous inhibitory transmission enhancement produced by melittin (1 μM) is not affected by 5-HT₃-receptor and P2X-receptor antagonists [ICS-205,930 (ICS, 2 nM) and PPADS (10 μM), respectively]. *A, B*: recordings of GABAergic sIPSCs in the absence and presence of melittin in Krebs solution containing ICS (*A*) or PPADS (*B*). *C*: the frequency (*left*) and amplitude (*right*) of GABAergic sIPSC under the action of melittin (measured around 3 min after the beginning of its superfusion) in the absence (*n = 32; these data were taken from Liu et al. 2008*) and presence of ICS (*n = 10*) or PPADS (*n = 6*), relative to those just before melittin superfusion [ICS: 1.3 ± 0.1 Hz and 11.2 ± 0.4 pA (*n = 10*); PPADS: 1.3 ± 0.1 Hz and 11.5 ± 0.2 pA (*n = 6*)]. Krebs solution contained strychnine (1 μM). $V_H = 0$ mV.

**FIG. 4.** Enhancements of GABAergic spontaneous inhibitory transmission by melittin (1 μM) and a nicotinic acetylcholine-receptor agonist (-)-nicotine (50 μM). *A, B*: recordings of GABAergic sIPSCs in the absence and presence of melittin (*A*) or (-)-nicotine (*B*). *A* and *B* were obtained from the same neuron; (-)-nicotine was
applied about 30 min after washout of melittin.  

C: the frequency (left) and amplitude (right) of GABAergic sIPSC under the action of melittin or (-)-nicotine (measured around 3 min after the beginning of its superfusion), relative to control [before melittin and (-)-nicotine application: 1.1 ± 0.1 Hz and 11.4 ± 0.6 pA (n = 9) and 1.3 ± 0.2 Hz and 12.5 ± 1.0 pA (n = 9), respectively]. Krebs solution contained strychnine (1 μM).  

$V_H = 0$ mV.

FIG. 5. Enhancements of GABAergic spontaneous inhibitory transmission by melittin (1 μM) and a muscarinic acetylcholine-receptor agonist carbamoylcholine (10 μM).  

A, B: recordings of GABAergic sIPSCs in the absence and presence of melittin (A) or carbamoylcholine (B).  

A and B were obtained from the same neuron; 

carbamoylcholine was applied about 30 min after washout of melittin.  

C: the frequency (left) and amplitude (right) of GABAergic sIPSC under the action of melittin or carbamoylcholine (measured around 3 min after the beginning of its superfusion), relative to control [before melittin and carbamoylcholine application: 3.1 ± 1.0 Hz and 11.0 ± 0.5 pA (n = 10) and 2.5 ± 0.3 Hz and 11.2 ± 0.6 pA (n = 10), respectively]. Krebs solution contained strychnine (1 μM).  

$V_H = 0$ mV.

FIG. 6. Enhancements of GABAergic spontaneous inhibitory transmission by melittin (1 μM) and norepinephrine (20 μM).  

A, B: recordings of GABAergic sIPSCs in the absence and presence of melittin (A) or norepinephrine (B).  

A and B were obtained from the same neuron; norepinephrine was applied about 30 min after washout of melittin.  

C: the frequency (left) and amplitude (right) of GABAergic sIPSC under the action of melittin or norepinephrine (measured around 3 min after the beginning of its superfusion), relative to control [before melittin and norepinephrine application: 2.8 ± 0.4 Hz and 11.9 ± 0.7 pA (n = 16) and 2.8 ± 0.6 Hz and 11.9 ± 0.7 pA (n = 16), respectively]. Krebs solution contained strychnine (1 μM).  

$V_H = 0$ mV.
FIG. 7. GABAergic spontaneous inhibitory transmission enhancement produced by melittin (1 μM) is slow in recovery after washout. A, B: recordings of GABAergic sIPSCs under the initial application of melittin (A) and 30 min after washout of melittin under its second application (B), where A and B were obtained from the same neuron. C, GABAergic sIPSC frequency (left) and amplitude (right) under the first (1st) and second applications (2nd) of melittin, relative to those [1st: 1.3 ± 0.1 Hz and 10.0 ± 0.1 pA (n = 6); 2nd: 1.3 ± 0.1 Hz and 10.5 ± 0.4 pA (n = 6)] before its application. Data obtained from the same neuron are connected by a straight line. Krebs solution contained strychnine (1 μM). V_H = 0 mV.

FIG. 8. The effect of the repeated application of (-)-nicotine (50 μM), carbamoylcholine (10 μM) or norepinephrine (20 μM) at a time interval of 30 min on GABAergic spontaneous inhibitory transmission. A, B, C: recordings of GABAergic sIPSCs under the first (upper) and second applications (lower) of (-)-nicotine (A), carbamoylcholine (B) or norepinephrine (C). Krebs solution contained strychnine (1 μM). V_H = 0 mV.

FIG. 9. Melittin (1 μM) enhances glycineergic but not GABAergic spontaneous inhibitory transmission in the presence of tetrodotoxin (TTX; 0.5 μM) and glutamate-receptor antagonists [non-NMDA receptor antagonist CNQX (10 μM) and NMDA receptor antagonist APV (50 μM)]. A: histograms of the half-width times of GABAergic (Aa) or glycineergic sIPSCs (Ab) in the control (black column) and under the action of melittin (gray column). Ba: recording of sIPSCs in the absence and presence of melittin in Krebs solution containing TTX, CNQX and APV. Lower traces, which are shown in an expanded scale in time, indicate sIPSCs recorded for a period indicated by a bar shown below the chart recording (upper). Note that the
number of GABAergic sIPSCs, indicated by arrow heads above the traces, does not increase under the action of melittin. *Bb*: histograms of the half-width times of GABAergic and glycinergic sIPSCs in the control (black column) and under the action of melittin (gray column). It is noted that melittin increased the event numbers of half-width times of sIPSCs corresponding to glycinergic (*Ab*) but not GABAergic ones (*Aa*). *Ba* and *Bb* were obtained from the same neuron. GABAergic and glycinergic sIPSCs in *A* were recorded in the presence of strychnine (1 μM) and bicuculline (10 μM), respectively, while sIPSCs in *B* were in the absence of both of the antagonists. 

\[ V_H = 0 \text{ mV}. \]