Phospholipase A₂ Activation Enhances Inhibitory Synaptic Transmission in Rat Substantia Gelatinosa Neurons

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Liu T, Fujita T, Nakatsuka T, Kumamoto E. Phospholipase A₂ (PLA₂) activation enhances inhibitory synaptic transmission in substantia gelatinosa neurons. J Neurophysiol 99: 1274–1284, 2008. First published January 23, 2008; doi:10.1152/jn.01292.2007. Phospholipase A₂ (PLA₂) activation enhances glutamatergic excitatory synaptic transmission in substantia gelatinosa (SG) neurons, which play a pivotal role in regulating nociceptive transmission in the spinal cord. By using melittin as a tool to activate PLA₂, we examined the effect of PLA₂ activation on spontaneous inhibitory postsynaptic currents (sIPSCs) recorded at 0 mV in SG neurons of adult rat spinal cord slices by use of the whole cell patch-clamp technique. Melittin enhanced the frequency and amplitude of GABAergic and glycinergic sIPSCs. The enhancement of GABAergic but not glycinergic transmission was largely depressed by Na⁺ channel blocker tetrodotoxin or glutamate-receptor antagonists (6-cyano-7-nitroquinoxaline-2,3-dione and/or DL-2-amino-5-phosphonovaleric acid) and also in a Ca²⁺-free Krebs solution. The effects of melittin on glycinergic sIPSC frequency and amplitude were dose-dependent with an effective concentration of ~0.7 μM for half-maximal effect and were depressed by PLA₂ inhibitor 4-bromophenacyl bromide or aristolochic acid. The melittin-induced enhancement of glycinergic transmission was depressed by lipoxigenase inhibitor nordihydroguaiaretic acid but not cyclooxygenase inhibitor indomethacin. These results indicate that the activation of PLA₂ in the SG enhances GABAergic and glycinergic inhibitory transmission in SG neurons. The former action is mediated by glutamate-receptor activation and neuronal activity increase, possibly the facilitatory effect of PLA₂ activation on excitatory transmission, whereas the latter action is due to PLA₂ and subsequent lipoxigenase activation and is independent of extracellular Ca²⁺. It is suggested that PLA₂ activation in the SG could enhance not only excitatory but also inhibitory transmission, resulting in the modulation of nociception.

INTRODUCTION

Phospholipase A₂ (PLA₂) is thought to play an important role in a variety of physiological functions including nociception through the production of arachidonic acid and subsequent synthesis of eicosanoids by actions of cyclooxygenase (COX) and lipoxigenase (LOX) (for review, see Dennis 1994; Shimizu and Wolfe 1990). There is much evidence supporting an involvement of COX metabolites prostanooids in enhancing nociceptive transmission in the spinal dorsal horn (for review, see Samad et al. 2002; Svensson and Yaksh 2002; Vanegas and Schaible 2001). For instance, Dirig and Yaksh (1999) have demonstrated that peripheral tissue injury and inflammation increase the release of prostanooids such as prostaglandin E₂ (PGE₂), possibly through PLA₂ activation, in the rat spinal cord. Baba et al. (2001) have reported that bath-applied PGE₂ produces a depolarization in dorsal horn neurons of rat spinal cord slices. Alternatively, it is possible that metabolites of LOX are involved in nociceptive transmission because the metabolites directly activate TRPV1, a molecule that plays a role in producing nociception (Hwang et al. 2000).

Melittin, a major component of bee venom, is a 26 amino-acid peptide that is known to have a variety of bioactive actions including PLA₂ activation and facilitation of synaptic transmission in the CNS (Aronica et al. 1992; Bernard et al. 1995; Phillis et al. 1999). We have recently reported that melittin reversibly enhances glutamatergic excitatory transmission in spinal dorsal horn substantia gelatinosa (SG) neurons (Yue et al. 2005), which play a crucial role in regulating nociceptive transmission to the CNS from the periphery (for review, see Willis and Coggeshall 1991). This action was due to PLA₂ activation without an involvement of arachidonic acid metabolites produced by COX and LOX (Yue et al. 2005). The SG neurons receive not only excitatory transmission but also GABAergic and glycinergic inhibitory transmission (Willis and Coggeshall 1991). There is much evidence showing that inhibitory transmission in the spinal dorsal horn may contribute to nociceptive transmission. For example, Moore et al. (2002) have reported an inhibition of primary-afferent-evoked inhibitory transmission in SG neurons and a reduction in the level of spinal dorsal horn GABA-synthesizing enzyme expression in partial nerve injury rat models compared with naive rats. Local blockade or knock-down of the potassium-chloride exporter KCC2 in the spinal dorsal horn, something that results in shift in transmembrane chloride gradient and thus causes normally inhibitory anionic synaptic currents to be excitatory, markedly reduced nociceptive thresholds in rats (Coull et al. 2003). To our knowledge, it has not been examined yet at the cellular level how PLA₂ activation itself affects inhibitory transmission in the spinal dorsal horn, although Ahmadi et al. (2002) have reported that PGE₂ blocks glycinergic transmission in rat superficial dorsal horn neurons (for review, see Zeilhofer 2005).

To know a role of PLA₂ in modulating inhibitory transmission at the spinal cord level, we examined the effect of melittin on spontaneous inhibitory postsynaptic currents (sIPSCs) in SG neurons of adult rat spinal cord slice preparations by using the whole cell patch-clamp technique.

METHODS

All animal experiments were approved by the Animal Care and Use Committee of Saga University.
Slice preparation

Spinal cord slices from adult rats were prepared as described previously (Fujita and Kumamoto 2006; Liu et al. 2004). In brief, male adult Sprague-Dawley rats (6–8 wk old) were anesthetized with urethane (1.2 g/kg ip), 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% O2-5% CO2, and maintained at 36 ± 1°C. The Krebs solution contained (in mM) 117 NaCl, 3.6 KCl, 2.5 CaCl2, 1.2 MgCl2, 11 glucose (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; Liu et al. 2004; Nakatsuka et al. 1999). Blind whole cell voltage-clamp recordings from SG neurons were made at a holding potential (Vh) of 0 mV where spontaneous glutamatergic excitatory postsynaptic currents (EPSCs) were invisible because the reversal potential for non-N-methyl-D-aspartate (non-NMDA) receptor channels involved in this production was close to 0 mV (Kohn et al. 1999; Yang et al. 2004) unless otherwise mentioned. Patch-pipettes were fabricated from thin-walled, fiber-filled capillary (1.5 mm OD) and contained the following solution (in mM): 110 Cs2SO4, 0.5 CaCl2, 2 MgCl2, 5 EGTA, 5 HEPES, 5 Mg-ATP, and 5 tetraethylammonium (TEA)-Cl (pH = 7.2). In some experiments, 110 mM-Cs2SO4 in the patch-pipette solution was replaced by 80 mM-CsCl, and 30 mM-CsCl, and the tonicity of this solution was adjusted by adding sucrose, which enabled to record sIPSCs having a considerable amplitude at −70 mV. Signals were acquired using an EPC-7 or Axopatch 200B amplifier. Currents obtained in the voltage-clamp mode were low-pass-filtered at 3 or 5 kHz and digitized at 333 or 500 kHz with an A/D converter (Digidata 1200 or 1322A). The data were stored and analyzed with a personal computer using pCLAMP v 9.2 software. The program (AxoGraph 4.0) 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 <50% of adjacent peaks.

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 tetrodotoxin (TTX; Wako, Osaka, Japan), melittin purified from bee venom, 4-bromophenacyl bromide (4-BPB), indomethacin (INDO), nordihydroguaiaretic acid (NDGA), strychnine nitrate, bicuculline methiodide, PLA2 from bee venom, aristrocotic acid, β-2-aminophosphonovaleric acid (APV; Sigma, St. Louis, MO), leukotriene B4 (LTB4; Cayman Chemicals, Ann Arbor, MI), and 6-cyano-7-nitroquinolinaline-2,3-dione (CNQX; Tocris Cookson, Bristol, UK). These drugs (except for melittin, PLA2, TTX, bicuculline, strychnine, and APV where distilled water was used as solvent) were first dissolved in dimethyl sulfoxide (DMSO) at 1000 (500 for IND and NDGA) 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. The toxicity of nominally Ca2+-free, high-Mg2+ (5 mM) Krebs solution was adjusted by lowering Na+ concentration of Krebs solution. When melittin (1 μM) was repeatedly superfused in the same spinal cord slice, time intervals between the application were >1 h.

Statistical analysis

Numerical data are presented as the means ± SE. Statistical significance was determined as P < 0.05 using either Student’s t-test (unless otherwise noted) or Kolmogorov-Smirnov 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, and their frequency and amplitude were not affected by a voltage-gated \(\text{Na}^{+}\) channel blocker TTX (data not shown), indicating that the sIPSCs occur in a manner independent of the spontaneous activity of neurons presynaptic to SG neurons (see Yang et al. 2004). The effects of melittin on the inhibitory transmission were examined in a total of 238 SG neurons.

Effect of melittin on inhibitory synaptic transmission in SG neurons

Melittin (1 \(\mu\)M) superfused for 3 min increased the frequency and amplitude of sIPSCs recorded at 0 mV in a reversible manner as seen in Fig. 1A. These actions were visible \(-2\) min after the beginning of melittin superfusion. Because melittin is known to produce a deteriorative effect on cell membranes (Fletcher and Jiang 1993), this peptide was not superfused for \(>3\) min at one time. When measured \(-3\) min after the beginning of its superfusion, sIPSC frequency and amplitude were, respectively, \(549 \pm 134\%\) \((n = 7; P < 0.05)\) and \(222 \pm 21\%\) \((n = 7; P < 0.05)\) of control (\(1.8 \pm 0.3\) Hz and \(14.2 \pm 2.2\) pA). Two types of GABAergic and glycinergic sIPSCs were encountered in SG neurons as reported previously (Kohno et al. 1999; Yang et al. 2004). A non-NMDA receptor antagonist CNQX (10 \(\mu\)M) did not affect GABAergic sIPSCs [amplitude and frequency: \(97 \pm 2\) and \(103 \pm 9\%\), respectively, of control \((9.8 \pm 0.9\) pA and \(2.1 \pm 0.3\) Hz); \(n = 4\)] and glycinergic sIPSCs [amplitude and frequency: \(101 \pm 3\) and \(106 \pm 10\%\), respectively, of control \((12.0 \pm 2.3\) pA and \(2.3 \pm 0.3\) Hz); \(n = 4\)], indicating no contamination of spontaneous EPSCs in sIPSCs. As seen in Fig. 1B, GABAergic sIPSCs, which were observed in the presence of a glycine-receptor antagonist strychnine (1 \(\mu\)M), were enhanced in frequency and amplitude by melittin (1 \(\mu\)M). Table 1 gives GABAergic sIPSC frequency and amplitude, measured \(-3\) min after the beginning of melittin superfusion, relative to control, which are obtained from 32 neurons. In the presence of a GABA_A-receptor antagonist bicuculline (10 \(\mu\)M), glycinergic sIPSCs, which were shorter in duration than GABAergic ones (compare sIPSC traces in expanded scale in time in Fig. 1, B and C), could be recorded. As seen for GABAergic sIPSCs, melittin (1 \(\mu\)M) increased glycinergic sIPSC frequency and amplitude (Fig. 1C). Table 1 shows the effects of melittin on glycinergic sIPSC frequency and amplitude, which are obtained from 27 neurons.

Similar actions of melittin on the frequency and amplitude of glycinergic sIPSCs recorded at \(-70\) mV were observed under the condition where a patch-pipette solution having 30 mM CsCl was used in Krebs solution containing CNQX (10 \(\mu\)M) and an NMDA receptor antagonist APV (50 \(\mu\)M). Around 3 min after the beginning of melittin (1 \(\mu\)M) superfusion, glycinergic sIPSC frequency and amplitude were, respectively, \(434 \pm 103\%\) \((n = 4; P < 0.05)\) and \(191 \pm 28\%\) \((n = 4; P < 0.05)\) of control (\(1.3 \pm 0.5\) Hz and \(7.2 \pm 0.4\) pA; data not shown). These percentage values were not significantly different from those obtained at 0 mV \((P > 0.05)\), indicating that the melittin action is independent of membrane potentials.

Melittin enhances GABAergic but not glycinergic transmission by facilitating glutamatergic transmission

To know whether the sIPSC frequency and amplitude increases produced by melittin are accompanied by an increase in neuronal activities, we next examined how the melittin-induced enhancement is affected by TTX (0.5 \(\mu\)M). Figures 2A and 3A demonstrate the effects of melittin (1 \(\mu\)M) on GABAergic and glycinergic transmission, respectively, in Krebs solution containing TTX. Melittin did not affect GABAergic miniature IPSC (mIPSC, i.e., sIPSC in the presence of TTX) frequency and amplitude while increasing glycinergic mIPSC ones. Figures 2C and 3C and Table 1 summarize the effects of melittin on GABAergic and glycinergic mIPSC frequency and amplitude, which are examined in 19 and 9 neurons, respectively. The GABAergic mIPSC frequency and amplitude was unaffected by melittin (Fig. 2C and Table 1). On the contrary, melittin increased glycinergic

<p>| Table 1. | Frequency and amplitude of GABAergic and glycinergic sIPSCs, relative to control |</p>
<table>
<thead>
<tr>
<th>Condition</th>
<th>Percentage of Control</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GABAergic sIPSC</strong></td>
<td>Frequency</td>
<td>Amplitude</td>
</tr>
<tr>
<td>Krebs solution</td>
<td>386 ± 37</td>
<td>178 ± 10</td>
</tr>
<tr>
<td>+ TTX</td>
<td>100 ± 4</td>
<td>97 ± 3</td>
</tr>
<tr>
<td>+ CNQX</td>
<td>272 ± 41</td>
<td>132 ± 13</td>
</tr>
<tr>
<td>+ CNQX + APV</td>
<td>145 ± 24</td>
<td>99 ± 8</td>
</tr>
<tr>
<td>Ca^{2+}-free Krebs solution</td>
<td>99 ± 5</td>
<td>93 ± 3</td>
</tr>
<tr>
<td><strong>Glycinergic sIPSC</strong></td>
<td>Frequency</td>
<td>Amplitude</td>
</tr>
<tr>
<td>Krebs solution</td>
<td>391 ± 36</td>
<td>207 ± 13</td>
</tr>
<tr>
<td>+ TTX</td>
<td>391 ± 75</td>
<td>195 ± 12</td>
</tr>
<tr>
<td>+ CNQX</td>
<td>381 ± 40</td>
<td>237 ± 22</td>
</tr>
<tr>
<td>+ CNQX + APV</td>
<td>366 ± 69</td>
<td>232 ± 40</td>
</tr>
<tr>
<td>+ 4-BPB (10 (\mu)M)</td>
<td>174 ± 21</td>
<td>177 ± 29</td>
</tr>
<tr>
<td>+ 4-BPB (50 (\mu)M)</td>
<td>141 ± 16</td>
<td>117 ± 10</td>
</tr>
<tr>
<td>+ aristolochic acid</td>
<td>166 ± 53</td>
<td>130 ± 28</td>
</tr>
<tr>
<td>+ INDO</td>
<td>305 ± 56</td>
<td>180 ± 26</td>
</tr>
<tr>
<td>+ NDGA</td>
<td>159 ± 27</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>Ca^{2+}-free Krebs solution</td>
<td>356 ± 55</td>
<td>171 ± 14</td>
</tr>
</tbody>
</table>

Frequency and amplitude were measured around 3 min after beginning of melittin (1 \(\mu\)M) superfusion under various conditions. Values are means ± SE.

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mIPSC frequency and amplitude; the extents of the increases were not significantly different from those for glycinergic sIPSCs (Fig. 3C).

No effect of melittin on GABAergic mIPSC frequency and amplitude suggests that melittin may have enhanced GABAergic transmission by the facilitation of excitatory transmission (Yue et al. 2005), which results in an increase in neuronal activities. To examine this possibility, next, we examined the effect of melittin (1 μM) on GABAergic transmission in Krebs solution containing CNQX (10 μM); data not shown. CNQX partially depressed the melittin-induced increases in GABAergic sIPSC frequency and amplitude; GABAergic sIPSC frequency and amplitude, measured around 3 min after the beginning of melittin superfusion, relative to control, which are obtained from ten neurons, are shown in Table 1. Adding APV (50 μM) to CNQX-containing Krebs solution resulted in blocking the facilitatory effect of melittin (1 μM) on GABAergic transmission, as seen in Fig. 2B; data obtained from 12 neurons are given in Fig. 2C and Table 1. Unlike GABAergic sIPSCs, glycinerigic sIPSCs were enhanced.

**FIG. 2.** GABAergic sIPSC frequency and amplitude is not facilitated by melittin in the presence of TTX or glutamate-receptor antagonists. A: recordings of GABAergic miniature IPSCs (mIPSCs) in the absence and presence of melittin (1 μM) in Krebs solution containing TTX (0.5 μM). B: recordings of GABAergic sIPSCs in the absence and presence of melittin (1 μM) in Krebs solution containing both 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and dl-2-amino-5-phosphonovaleric acid (APV, 50 μM). C: summary of GABAergic sIPSC frequency (left) and amplitude (right) under the action of melittin in the absence (n = 32) and presence of TTX (n = 19) or both CNQX and APV (n = 12), relative to control. In this and subsequent figures, vertical lines accompanied by bars indicate SE; statistical significance between data shown by bars is indicated by an asterisk, *: P < 0.05; n.s.: not significant. 

**FIG. 3.** Glycinergic sIPSC frequency and amplitude is facilitated by melittin in the presence of TTX or glutamate-receptor antagonists to an extent similar to that in the absence of the drugs. A: recordings of glycinerigic mIPSCs in the absence and presence of melittin (1 μM) in Krebs solution containing TTX (0.5 μM). B: recordings of glycinerigic sIPSCs in the absence and presence of melittin (1 μM) in Krebs solution containing both CNQX (10 μM) and APV (50 μM). Note in A and also in Fig. 5A that bicuculline itself produces a small inward current, as reported by Ataka and Gu (2006). C: summary of glycinerigic sIPSC frequency (left) and amplitude (right) under the action of melittin in the absence (n = 27) and presence of TTX (n = 9) or both CNQX and APV (n = 4), relative to control. 

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in frequency and amplitude by melittin (1 μM) in Krebs solution containing only CNQX (10 μM; n = 7) or both CNQX (10 μM) and APV (50 μM; n = 4; see Fig. 3B and Table 1). There was not a significant difference in glycinergic sIPSC frequency and amplitude increases produced by melittin between the presence and absence of extracellular Ca\(^{2+}\) (Fig. 4Bb).

In 47% of 88 SG neurons tested, bicuculline (10 μM) itself produced an inward current, as seen in Fig. 3A; the inward current had a peak amplitude of 11.8 ± 1.0 pA (n = 41), and the other neurons had no inward current. On the contrary, strychnine (1 μM) did not change holding currents (n = 43). Ataka and Gu (2006) have reported similar observations in mouse SG neurons and attributed the inward current to a vesicular release of GABA from nerve terminals.

### Characterization of the glycinergic transmission enhancement produced by melittin

Because no effects of TTX, glutamate-receptor antagonists and nominally Ca\(^{2+}\)-free solution on melittin-induced glycinergic transmission enhancement suggest a direct action of melittin on glycinergic transmission, we next characterized the glycinergic transmission enhancement produced by melittin. Figure 5, A and B demonstrates the time course of a change in glycinergic sIPSC frequency and amplitude following superfusion of melittin (1 μM). Both of its frequency and amplitude increased with a delay of 1–2 min after the beginning of melittin superfusion, and these increases subsided within 10 min after washout. Figure 5C demonstrates cumulative distributions of the inter-event interval and amplitude of glycinergic sIPSC in the control and under the effect of melittin. Melittin significantly increased the proportion of glycinergic sIPSCs having a shorter inter-event interval and of those having a larger
amplitude; this effect was confirmed in three other neurons. Figure 5 demonstrates an average of glycinergic sIPSC traces in the absence and presence of melittin in the same neuron as that of Fig. 5, A–C, and their superimposition where control glycinergic sIPSC trace is scaled in amplitude to that under the effect of melittin. As judged from this superimposition, the glycinergic sIPSC amplitude increase produced by melittin was not accompanied by a change in its decay phase. When examined in four neurons, the half-decay times of glycinergic sIPSC in the absence and presence of melittin were 3.7 ± 0.3 and 3.8 ± 0.3 ms, respectively; they were not significantly different (P > 0.05). Figure 6 demonstrates the effect of a repeated application of melittin (1 µM) on glycinergic transmission in the same neuron. When melittin was once again applied 30 min after its washout, the effects of the second application of melittin on glycinergic sIPSC frequency and amplitude (Fig. 6B) were much smaller in extent than those of the first application of melittin (Fig. 6A). Figure 6C shows a comparison of relative frequency and amplitude of glycinergic sIPSC under the action of melittin to those before its superfusion between the first and second applications, which was obtained from six neurons. When examined in different SG neurons in the same slice preparation 1 h after washout of melittin (1 µM), the facilitatory effect on glycinergic transmission of the second application of melittin was not distinct from that of its first application over a variation of sIPSC frequency and amplitude increases among neurons (data not shown).

Figure 7A illustrates the effect on glycinergic transmission of melittin in a concentration range of 0.2–1 µM. With an increase in melittin concentrations, its facilitatory effect became fast in onset and increased in extent. Figure 7B demonstrates dose-response relationships for glycinergic sIPSC frequency and amplitude increases produced by melittin in a range of 0.1–5 µM, obtained from 4 to 27 neurons. Analysis based on the Hill equation showed that the effective concentrations of melittin for half-maximal effect (EC$_{50}$s) in increasing glycinergic sIPSC frequency and amplitude are 0.73 and 0.64 µM, respectively. Here melittin at concentrations >5 µM was not used, because melittin (10 µM) reportedly produces a noticeable damage to synaptosomal membranes (González et al. 1997).
Because melittin is known to have effects other than PLA2 activation (for review, see Fletcher and Jiang 1993), first we examined whether the glycineric transmission enhancements produced by melittin are really mediated by PLA2 by using a PLA2 inhibitor 4-BPB (Mayer and Marshall 1993). The pretreatment for 4 min with 4-BPB [10 μM, a concentration used previously by us to examine an involvement of PLA2 in the melittin-induced enhancement of glutamatergic transmission (Yue et al. 2005)] partially inhibited the effects of melittin (1 μM) on glycineric sIPSC frequency and amplitude; the frequency and amplitude around 3 min after the beginning of melittin superfusion, relative to control, which was obtained from eight cells, is given in Table 1. This concentration of 4-BPB may not have been enough to inhibit the melittin-induced enhancement of glycineric transmission because this enhancement is much larger in extent than that of glutamater-
mission; the frequency and amplitude of the sIPSC were, respectively, 101 ± 8% (n = 3; P > 0.05) and 100 ± 5% (n = 3; P > 0.05) of control (2.7 ± 0.6 Hz and 14.1 ± 1.5 pA) around 4 min after the beginning of its superfusion.

Because one of LOX metabolites, LTB4, activates TRPV1 (Hwang et al. 2000) and increases intracellular Ca2+ concentrations in dorsal root ganglion neurons (Andoh and Kuraishi 2005), LTB4 was tested as a candidate of endogenous substances involved in the enhancement of glycinergic transmission as a result of PLA2 activation. LTB4 at 0.1 μM increased glycinergic transmission [frequency and amplitude of the sIPSC: 99 ± 8% (n = 3; P > 0.05) and 100 ± 2% (n = 3; P > 0.05), respectively, of control (2.6 ± 0.7 Hz and 12.5 ± 1.4 pA) ~3 min after the beginning of LTB4 superfusion; data not shown]. In SG neurons where melittin (1 μM) increased glycinergic sIPSC frequency and amplitude [329 ± 21% (n = 3; P < 0.05) and 201 ± 39% (n = 3; P < 0.05), respectively, of control (2.6 ± 0.5 Hz and 14.4 ± 1.9 pA) around 3 min after the beginning of melittin superfusion], LTB4 superfused at 0.5 μM before the application of melittin did not affect them [frequency and amplitude: 107 ± 8% (n = 3; P > 0.05) and 98 ± 12% (n = 3; P > 0.05), respectively, of control (2.4 ± 0.3 Hz and 14.8 ± 1.9 pA) ~3 min after the beginning of LTB4 superfusion; data not shown].

**FIG. 7.** Melittin dose-dependently increases glycinergic sIPSC frequency and amplitude in SG neurons. A: chart recordings of glycinergic sIPSCs in the absence and presence of melittin at concentrations of 0.2, 0.5, and 1 μM. B: dose-response curves for glycinergic sIPSC frequency (top) and amplitude (bottom) under the effect of melittin, relative to control. The value in parentheses denotes the number of cells examined. The dose-response curves were drawn according to the Hill equation (frequency: EC50 = 0.73 μM, Hill coefficient: 1.69; amplitude: EC50 = 0.64 μM, Hill coefficient: 1.96). Krebs solution contained bicuculline (10 μM). Vh = 0 mV.

**DISCUSSION**

The present study demonstrated that melittin increases the frequency and amplitude of glycinergic and GABAergic sIPSCs in all of SG neurons tested although the SG is comprised of a heterogenous cell group (Grudt and Perl 2002); the enhancement by melittin of glutamatergic spontaneous excitatory transmission in SG neurons has been previously reported by Yue et al. (2005). The glycinergic transmission enhancement produced by melittin was resistant to TTX and glutamate-receptor antagonists (CNQX and APV), indicating a direct action of melittin on glycinergic transmission. On the other hand, the GABAergic transmission enhancement produced by melittin was not seen in the presence of TTX or glutamate-receptor antagonists and in a Ca2+ -free Krebs solution, indicating an involvement of the facilitatory effect of melittin on excitatory transmission and of an increase in neuronal activities in the SG.

**Enhancement by melittin of glycinergic inhibitory transmission in SG neurons**

The melittin-induced enhancement of glycinergic transmission is both pre- and postsynaptic in origin because melittin increases the proportion of glycinergic sIPSCs having a shorter inter-event interval and of those having a larger amplitude. Consistent with this presynaptic action, Phillis et al. (1999) have reported that melittin enhances the release of glycine from the rat cerebral cortex in vivo. EC50 values for the pre- and postsynaptic actions in SG neurons were, respectively, 0.73 and 0.69 μM, values comparable to that (1.1 μM) for the presynaptic action at glutamatergic synapses in the SG (Yue et al. 2005). These values were larger by about sevenfold than that (0.11 μM) in enhancing aspartate release from cultured neurons (Aronica et al. 1992) while being smaller by about fivefold than that (3.5 μM) in increasing AMPA receptor affinity (Bernard et al. 1995). The glycinergic sIPSC amplitude increase produced by melittin is not accompanied by a change in the half-decay time of the sIPSC. This effect may be due to
a change not in the channel kinetics of the glycine receptor but in its affinity for glycine because the decay phase of glycineergic synaptic current in spinal cord neurons is thought to be produced by a random closure of individual glycine-receptor channels (see Takahashi and Momiyama 1991). With respect to this postsynaptic effect, a rise in intracellular Ca$^{2+}$ being involved in increasing an apparent affinity of the glycine receptor for glycine as a result of melittin actions (see Fucile et al. 2000).

Melittin did not significantly affect glycineergic transmission in the presence of 4-BPB or aristolochic acid, suggesting an involvement of PLA$_2$ activation. In support of this idea, the spinal cord contains secreted and cytosolic PLA$_2$ (Samad et al. 2001; for review, see Svensson and Yaksh 2002). Although it is known that arachidonic acid produced as a result of PLA$_2$ actions can enhance glycinergic transmission, melittin-mediated enhancement does not require this metabolite. Instead, melittin enhances glycineergic transmission by activating lipoxygenase (LOX) but not cyclooxygenase (COX) metabolites of arachidonic acid (Fig. 9).
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activation is metabolized by various enzymes including COX and LOX (Shimizu and Wolfe 1990), the disappearance of this melittin effect in the presence of NDGA but not INDO suggests an involvement of the metabolites of LOX but not COX. The lack of the effect of INDO on the melittin-induced increase in glycnergic sIPSC amplitude suggests that an inhibitory effect of PGE₂ on glycine-receptor responses in rat superficial dorsal horn neurons (Ahmadi et al. 2002; for review, see Zeilhofer 2005) may have not contributed to the effect of PLA₂ activation on glycnergic sIPSC amplitudes in the present study, probably because of an insufficient expression of COX and PGE synthase such as microsomal PGE₂ synthase-1 (Guay et al. 2004), both of which are required for spinal PGE₂ production (for review, see Shimizu and Wolfe 1990). Consistent with an involvement of such a metablitie system, a repeated application of melittin at 30-min interval had almost no effects on glycnergic transmission. This was so even when glycnergic sIPSC frequency and amplitude recovered to those before the application of melittin. An intracellular system leading to the enhancement of glycnergic transmission may exhibit a desensitization. As the melittin-induced enhancement of glycnergic transmission persists in a Ca²⁺-free Krebs solution, this effect is independent of extracellular Ca²⁺. Because one of LOX metabolites, LTB₄, does not affect glycnergic transmission in SG neurons, other LOX metabolites than LTB₄ appear to be involved in the glycnergic transmission enhancement produced by PLA₂ activation. Intracellular mechanisms for glycnergic transmission enhancement following PLA₂ activation remain to be examined further.

Enhancement by melittin of glycnergic inhibitory transmission in SG neurons

The melittin-induced enhancement of glycnergic transmission may be mediated by neumodulators released from a neuron as a result of excitatory transmission enhancement caused by PLA₂ activation and subsequent increase in neuronal activities because the former action is resistant to TTX (see Yue et al. 2005), and thus the enhancement is expected to produce action potentials. In support of this idea, it is known that many kinds of neumodulators enhance glycnergic inhibitory transmission in SG neurons. For instance, acetylcholine enhances inhibitory transmission in SG neurons by activating nicotinic and muscarinic acetylcholine receptors (Baba et al. 1998; Takeda et al. 2003). Norepinephrine increases the amplitude and frequency of glycnergic sIPSC in SG neurons (Baba et al. 2000b). It remains to be examined what kinds of neuromodulators are involved in the glycnergic transmission enhancement produced by PLA₂ activation.

It is of interest to note that glycnergic but not GABAergic transmission is presynaptically affected by PLA₂ activation. This suggests that GABA and glycine may be released from a different nerve terminal containing either GABA or glycine as different from the case in the lamina I where they are thought to be co-released from a single nerve terminal (Chéry and de Koninck 1999). This may be consistent with the observations that some presynaptic terminals in the SG contain GABA without glycine (Spike and Todd 1992) and that glycnergic transmission is affected by norepinephrine in a distinct manner from that of glycnergic transmission in SG neurons (Baba et al. 2000a). The presence of a tonic GABA but not glycine current may be in part due to the fact that GABA and glycine are released from nerve terminals different from each other.

Physiological significance of PLA₂ activation in the SG

The present study together with our previous one (Yue et al. 2005) revealed that PLA₂ activation in the SG enhances glycnergic inhibitory and glutamatergic excitatory transmission, the latter of which actions leads to the enhancement of GABAergic inhibitory transmission. Neumodulators such as opioids at the spinal dorsal horn level generally hyperpolarize membranes and inhibit glutamatergic transmission in SG neurons (Kohno et al. 1999; Fujita and Kumamoto 2006; for review, see Fürst 1999), both of which reduce the excitability of the neurons, an effect of the enhancement of glycnergic transmission. Neuromodulators such as acetylcholine and nor-epinephrine, which enhance GABAergic transmission in SG neurons, act as neumoduges when administered intratheceally (Abram and O’Connor 1995; Abram and Winne 1995; Howe et al. 1983; Khan et al. 1998, 2001; Reddy et al. 1980). Thus PLA₂ activation in the SG may inhibit nociceptive transmission from the periphery in a complex manner. Although Young et al. (1995) have suggested that PLA₂ may be involved in a sustained increased activity of spinal dorsal horn neurons in response to a repeated application of mustard oil to the periphery, PLA₂ activation would enhance not only excitatory but also inhibitory transmission in spinal dorsal horn neurons. Thus inflammation activates PLA₂ and then induces pain sensitization (Dirig and Yaksh 1999), while PLA₂ activation should enhance glycnergic transmission in SG neurons, an anti-hyperalgesic effect as shown in the present study. Taken into consideration that the glycnergic transmission enhancement is mediated by LOX but not COX metabolites, COX inhibitors given during inflammatory hyperalgesia (for instance, see Zhang et al. 1997) might shift the metabolism of arachidonic acid from COX to LOX metabolites, which could then contribute to the analgesic action of the COX inhibitors.

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