Proteinase-Activated Receptor-1 Activation Presynaptically Enhances Spontaneous Glutamatergic Excitatory Transmission in Adult Rat Substantia Gelatinosa Neurons

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Fujita T, Liu T, Nakatsuka T, Kumamoto E. Proteinase-activated receptor-1 activation presynaptically enhances spontaneous glutamatergic excitatory transmission in adult rat substantia gelatinosa neurons. J Neurophysiol 102: 312–319, 2009. First published May 6, 2009; doi:10.1152/jn.91117.2008. Proteinase-activated receptors (PARs) have a unique activation mechanism in that a proteolytically exposed N-terminal region acts as a tethered ligand. A potential impact of PAR on sensory processing has not been fully examined yet. Here we report that synthetic peptides with sequences corresponding to PAR ligands enhance glutamatergic excitatory transmission in substantia gelatinosa (SG) neurons of adult rat spinal cord slices by using the whole cell patch-clamp technique. The frequency of spontaneous excitatory postsynaptic current (EPSC) was increased by PAR-1 agonist SFLLRN-NH₂ (by 47% at 1 μM) with small increases by PAR-2 and -4 agonists (SLIGKV-NH₂ and GYPGQV-OH, respectively; at >3 μM); there was no change in its amplitude or in holding current at −70 mV. The PAR-1 peptide action was inhibited by PAR-1 antagonist YFLLRN-P-OH. TFLLR-NH₂, an agonist which is more selective to PAR-1 than SFLLRN-NH₂, dose-dependently increased spontaneous EPSC frequency (EC₅₀ = 0.32 μM). A similar presynaptic effect was produced by PAR-1 activating proteinase thrombin in a manner sensitive to YFLLRN-P-OH. The PAR-1 peptide action was resistant to tetrodotoxin and inhibited in Ca²⁺-free solution. Primary-afferent monosynaptically evoked EPSC amplitudes were unaffected by PAR-1 agonist. These results indicate that PAR-1 activation increases the spontaneous release of L-glutamate onto SG neurons by PAR-1 agonist. These results indicate that PAR-1 activation presynaptically enhances spontaneous glutamatergic excitatory transmission in adult rat substantia gelatinosa neurons. J Neurophysiol 102: 312–319, 2009. First published May 6, 2009; doi:10.1152/jn.91117.2008. Proteinase-activated receptors (PARs) have a unique activation mechanism in that a proteolytically exposed N-terminal region acts as a tethered ligand. A potential impact of PAR on sensory processing has not been fully examined yet. Here we report that synthetic peptides with sequences corresponding to PAR ligands enhance glutamatergic excitatory transmission in substantia gelatinosa (SG) neurons of adult rat spinal cord slices by using the whole cell patch-clamp technique. The frequency of spontaneous excitatory postsynaptic current (EPSC) was increased by PAR-1 agonist SFLLRN-NH₂ (by 47% at 1 μM) with small increases by PAR-2 and -4 agonists (SLIGKV-NH₂ and GYPGQV-OH, respectively; at >3 μM); there was no change in its amplitude or in holding current at −70 mV. The PAR-1 peptide action was inhibited by PAR-1 antagonist YFLLRN-P-OH. TFLLR-NH₂, an agonist which is more selective to PAR-1 than SFLLRN-NH₂, dose-dependently increased spontaneous EPSC frequency (EC₅₀ = 0.32 μM). A similar presynaptic effect was produced by PAR-1 activating proteinase thrombin in a manner sensitive to YFLLRN-P-OH. The PAR-1 peptide action was resistant to tetrodotoxin and inhibited in Ca²⁺-free solution. Primary-afferent monosynaptically evoked EPSC amplitudes were unaffected by PAR-1 agonist. These results indicate that PAR-1 activation increases the spontaneous release of L-glutamate onto SG neurons by PAR-1 agonist. These results indicate that PAR-1 activation presynaptically enhances spontaneous glutamatergic excitatory transmission in adult rat substantia gelatinosa neurons. J Neurophysiol 102: 312–319, 2009. First published May 6, 2009; doi:10.1152/jn.91117.2008. Proteinase-activated receptors (PARs), which are a family of G-protein-coupled receptors and are activated as a result of the cleavage of their N-terminus by serine proteinases such as thrombin, are thought to play an important role in a variety of physiological and pathological phenomena including blood coagulation, inflammation, gastrointestinal function, and pain (for review, see Cenac and Vergnolle 2005; Gingrich and Traynelis 2000; Noorbakhsh et al. 2003; Ossovskaya and Bunnett 2004). Molecular cloning has revealed four members of the PAR family, PAR-1 to -4 (Noorbakhsh et al. 2003). The PAR-1-mediated glutamatergic transmission enhancement could be involved in a positive modulation of nociceptive transmission. INTRODUCTION

Proteinase-activated receptors (PARs), which are a family of G-protein-coupled receptors and are activated as a result of the cleavage of their N-terminus by serine proteinases such as thrombin, are thought to play an important role in a variety of physiological and pathological phenomena including blood coagulation, inflammation, gastrointestinal function, and pain (for review, see Cenac and Vergnolle 2005; Gingrich and Traynelis 2000; Noorbakhsh et al. 2003; Ossovskaya and Bunnett 2004). Molecular cloning has revealed four members of the PAR family, PAR-1 to -4 (Noorbakhsh et al. 2003). The PAR-1 has a unique activation mechanism in that a proteolytically exposed N-terminal region acts as a tethered ligand that binds to a site on the receptor itself; this results in triggering a signal transduction cascade. In the absence of proteinases, PARs, except for PAR-3, can be activated by synthetic peptides with sequences corresponding to the PAR-tethered ligands (Ossovskaya and Bunnett 2004; Vu et al. 1991). In the nervous system, activation of PARs alters the morphology of neurons and astrocytes, and produces glial cell proliferation in the CNS (Grabham and Cunningham 1995; Jalink and Moolenaar 1992; Striggow et al. 2001; for review, see Wang and Reiser 2003). Moreover, there is evidence supporting the role of PARs at synapse levels. For instance, PAR ligand peptides presynaptically inhibit cholinergic fast excitatory transmission in guinea pig small intestine myenteric neurons (Gao et al. 2002) and depolarize membranes of guinea pig ileal and small intestine myenteric neurons (Gao et al. 2002; Lindén et al. 2001). PAR-1 activation potentiates N-methyl-D-aspartate (NMDA)-receptor responses in hippocampal CA1 pyramidal neurons (Gingrich and Traynelis 2000; Gingrich et al. 2000). Although PARs are thus involved in modulating synaptic transmission in the nervous system, this involvement has not been fully examined yet in potential impact on sensory processing (see Ossovskaya and Bunnett 2004; Wang and Reiser 2003).

All of the PARs are expressed in the adult rat dorsal root ganglia (DRG) (Dai et al. 2004; de Garavilla et al. 2001; Steinhoff et al. 2000; Zhu et al. 2005) and PAR-1 and -2 appear to be involved in nociceptive transmission in peripheral terminals of primary afferents (Asfaha et al. 2002; Dai et al. 2004; Kawabata et al. 2002; Steinhoff et al. 2000; Zhu et al. 2005). It is possible that PARs are expressed in not only peripheral but also central terminals of primary afferents, and also in the spinal dorsal horn, and thus are involved in the modulation of synaptic transmission there. To address this issue, we examined whether PAR ligand peptides affect glutamatergic excitatory synaptic transmission in substantia gelatinosa (SG; lamina II of Rexed) neurons that are thought to play a pivotal role in regulating nociceptive transmission in the spinal dorsal horn (Willis and Coggeshall 1991). A part of the present results has been reported in abstract form (Fujita et al. 2007).

METHODS

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

Adult rat spinal cord slice preparations without dorsal roots were obtained in a manner similar to that described previously (Fujita and Kumamoto 2006). In brief, adult Sprague-Dawley rats (6–8 wk old; 200–300 g) were anesthetized with urethane (1.5 g/kg body wt ip), and a laminectomy was performed to extract a lumbarosacral spinal cord segment. In some experiments, adult rat spinal cord slices that retained an attached dorsal root were obtained, as reported previously (Ataka et al. 2000; Lao et al. 2004; Nakatsuka et al. 2000). The spinal cord was quickly immersed in ice-cold (1–3°C) Krebs solution (in mM: 117 NaCl, 3.6 KCl, 1.2 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgCl₂, 25 NaHCO₃, and 11 glucose) bubbled with 95% O₂-5% CO₂. Rats were killed by exsanguination. A transverse slice (600–650 μm thick) without or with a dorsal root was cut using a microslicer (DTK-1000, Dousaya, Kyoto, Japan) in oxygenated ice-cold Krebs solution. The slice was then transferred to the recording chamber (volume: 1.5 ml), and continuously perfused with preheated (35 ± 1°C) and oxygenated Krebs solution for ≥1 h before recordings.

Whole cell voltage-clamp recordings

The SG can be identified under a stereomicroscope as a translucent band across the dorsal horn (Fujita and Kumamoto 2006). Spinal cord slices could be maintained for >12 h when they were superfused at 10–15 ml/min with preoxygenated Krebs solution at 35 ± 1°C. The recorded neurons were located at the center of SG to avoid recordings from laminae I and III neurons. Patch-pipettes were filled with solution (in mM) containing 135 K-gluconate, 5 KCl, 0.5 CaCl₂, 2 MgCl₂, 5 EGTA, 5 HEPES, and 5 Mg-ATP (pH 7.2) and had a resistance of 8–18 MΩ. The holding potential (Vh) used to record excitatory postsynaptic currents (EPSCs) was −70 mV, as reported previously (Fujita and Kumamoto 2006). Aβ- and C-fiber evoked EPSCs were elicited by stimulating the dorsal root, as mentioned previously (Ataka et al. 2000; Nakatsuka et al. 2000). In brief, the stimulation was performed by using a suction electrode with a constant current source of pulse at a frequency of 0.1 Hz unless otherwise mentioned. The strength of stimuli (duration: 0.1 ms) used was 1.2 times a threshold to elicit EPSCs fearing a conduction block of action potentials in the dorsal root. Their evoked EPSCs were distinguished from each other, based on a minimal stimulus strength enough to elicit the EPSCs and a latency of the EPSCSs. C-fiber EPSCs required more larger stimulus intensity for the activation than Aβ-fiber EPSCs and Aδ-fiber EPSCs had a shorter latency than C-fiber EPSCs. Aδ-fiber EPSCs were judged to be monosynaptic when the latency remained constant and there was no failure during stimulation at 20 Hz for 1 s, while C-fiber ones were so when failures did not occur during repetitive stimulation at 1 Hz for 20 s as done previously (Ataka et al. 2000; Nakatsuka et al. 2000). Conduction velocities (CVs) of the afferent fibers were calculated from the latency of monosynaptic EPSC and the length of dorsal root (see Ataka et al. 2000; Lao et al. 2004; Nakatsuka et al. 2000). Signals were acquired using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Currents obtained in the voltage-clamp mode were low-pass-filtered at 3 kHz, and digitized at 500 kHz with an A/D converter (Digidata 1322A, Molecular Devices). The data were stored and analyzed with a personal computer using pCLAMP 9.2 software (Molecular Devices).

Data analysis

Many of spontaneous events were detected and analyzed using Mini Analysis Program version 5.0.3 (Synaptosoft, Decatur, GA); detection criteria for sEPSCs included a 5-pA event threshold, their fast rise time and a decay curve that approximated to an exponential decay. AxoGraph 4.0 (Molecular Devices) was also used, as reported previously (Fujita and Kumamoto 2006); the results so obtained were not different from those obtained by using Mini Analysis Program. Numerical data are presented as the means ± SE, and statistical significance was set at P < 0.05 using a paired Student’s t-test (unless otherwise mentioned) or a Kolmogorov-Smirnov test. In all cases, n refers to the number of neurons studied.

Application of drugs

All drugs were applied by switching the perfusion solution to one containing the drug at a known concentration using a three-way tap. The perfusion rate or temperature was not altered. Drug-containing solutions reached the recording chamber within 8 s. Drugs used were SFLLRN-NH₂ (SFLLRN), SLIGKV-NH₂ (SLIGKV), SLIGRL-NH₂ (SLIGRL), TFRGAP-OH (TFRGAP), GYPGQV-OH (GYPGQV), YFLLRN-OH (YFLLRN), and TFLLRN-NH₂ (TFLLRN) from Bachem (Bubendorf, Switzerland); 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) from Tocris Neuramin (Bristol, UK); tetrodotoxin (TTX) from Wako (Osaka, Japan); and human plasma thrombin from Sigma (St. Louis, MO). All drugs except for CNQX (where dimethyl sulfoxide was used as a solvent) and thrombin were first dissolved in distilled water at 1,000 times the concentration to be used and then diluted to the desired concentration in Krebs solution immediately before use. Thrombin was directly dissolved in Krebs solution. The toxicity of nominally Ca²⁺-free, high-Mg²⁺ (5 mM) Krebs solution was adjusted by lowering Na⁺ concentration of Krebs solution.

RESULTS

SG neurons had resting membrane potentials more negative than −50 mV (when measured in a current-clamp mode), and exhibited at a Vh of −70 mV glutamatergic spontaneous EPSCs (sEPSCs) which were blocked by a non-NMDA receptor antagonist CNQX (10 μM; data not shown). No spontaneous inhibitory postsynaptic currents were observed at this Vh because their reversal potential was near −70 mV. The frequency and amplitude of sEPSCs was unaffected by a voltage-gated Na⁺ channel blocker TTX (0.5 μM; data not shown), as reported previously (Fujita and Kumamoto 2006; Yue et al. 2005), indicating that the production of the sEPSCs was independent of the spontaneous TTX-sensitive activity of neurons presynaptic to SG neurons.

In all of SG neurons examined, superfusing a PAR-1 ligand peptide SFLLRN (Vergnolle et al. 2001) at a concentration of 1 μM for 5 min reversibly increased the frequency of the sEPSC with a change in the amplitude or in holding current at −70 mV, as seen in Fig. 1A. This increase in sEPSC frequency was visible around 2 min after the beginning of its superfusion and thereafter attained a maximal effect. The frequency and the amplitude of sEPSCs peaked at 5 min after the beginning of SFLLRN superfusion, respectively, 146 ± 7% (n = 27; P < 0.05) and 102 ± 6% (n = 27; P > 0.05) of control (see Fig. 3C). Such a facilitatory effect of SFLLRN subsided within 5 min after washout. Figure 1B demonstrates averages of the time courses of changes in sEPSC frequency and amplitude under the actions of SFLLRN, relative to control, which are obtained from 24 to 25 neurons. When the effect of SFLLRN on cumulative distributions of the inter-event interval and amplitude of sEPSC was examined in a single neuron, SFLLRN shifted the distribution of sEPSC inter-event interval to left (P < 0.001) without a change in sEPSC amplitude distribution (P > 0.05; Kolmogorov-Smirnov test), as shown in Fig. 1C; this result was confirmed in three other neurons.
Unlike PAR-1 ligand, SLIGKV and GYPGQV, which are, respectively, synthetic peptides with sequences corresponding to PAR-2 and -4 tethered ligands (Vergnolle et al. 2001), at 1/μM did not affect spontaneous excitatory transmission, as seen in Fig. 2, Aa and Ba. When tested at higher concentrations such as 3–5 μM, SLIGKV and GYPGQV enhanced the frequency but not amplitude of sEPSC; these extents were minimal but significant (see Fig. 2, Ab and Bb). TFRGAP (1 μM), which is a synthetic peptide with a sequence corresponding to PAR-3-tethered ligand albeit being not a PAR-3 agonist, also did not affect sEPSC frequency and amplitude; they were 99 ± 1% (P > 0.05) and 101 ± 4% (P > 0.05), respectively, of control (8.1 ± 1.7 Hz and 13.7 ± 1.1 pA; n = 14) ~3.5 min after the beginning of its superfusion (data not shown). These peptides used in this study correspond to tethered ligands for human PARs. A rat PAR-2 ligand peptide SLIGRL (1 μM), which is similar in sequence to human one (SLIGKV) (Vergnolle et al. 2001), also did not affect spontaneous excitatory transmission; sEPSC frequency and amplitude ~3.5 min after the beginning of this superfusion were, respectively, 105 ± 16% (P > 0.05)
and 102 ± 3% (P > 0.05) of control (9.3 ± 2.6 Hz and 15.1 ± 2.9 pA; n = 4; data not shown).

Because SFLLRN may act on PARs other than PAR-1, we next examined whether or not the effect of SFLLRN (1 μM) on spontaneous excitatory transmission is affected by pretreatment with a PAR-1 antagonist peptide YFLLRP (1 μM) (Rasmussen et al. 1993) for 4 min. As shown in Fig. 3A, the SFLLRN-induced increase in sEPSC frequency was reduced in extent in the presence of YFLLRN. Figure 3B demonstrates averages of the time courses of changes in sEPSC frequency and amplitude under the action of YFLLRN and then of SFLLRN in the presence of YFLLRN, relative to those before YFLLRN superfusion, which are obtained from seven to eight neurons. SFLLRN-induced increase in sEPSC frequency ∼3.5 min after the beginning of its superfusion in the presence of YFLLRN was significantly smaller than that in the absence of YFLLRN (see Fig. 3C). Altogether, these results indicate that PAR-1 participates in the SFLLRN effect. Although YFLLRN was reported to be a partial agonist of PAR-1 (Rasmussen et al. 1993), this peptide (1 μM) by itself, in the present study, did not affect sEPSC frequency and amplitude; they were, respectively, 100 ± 3% (P > 0.05) and 100 ± 3% (P > 0.05) of control (6.9 ± 0.9 Hz and 11.9 ± 1.0 pA; n = 4) ~3.5 min after the beginning of YFLLRN superfusion. YFLLRN at a high concentration (5 μM) also did not affect spontaneous excitatory transmission [sEPSC frequency and amplitude: 109 ± 6% (P > 0.05) and 105 ± 9% (P > 0.05) of control, respectively; n = 4].

The possible involvement of PAR-1 in sEPSC frequency increase was further examined by using a peptide agonist TFFLR, which is more selective for PAR-1 than SFLLRN because SFLLRN is reported to activate not only PAR-1 but also PAR-2 (Vergnolle et al. 2001). Like SFLLRN, TFFLR at a concentration of 1 μM enhanced the frequency of sEPSC without a change in its amplitude, as seen in Fig. 4A. The frequency and the amplitude of sEPSC ∼3.5 min after the beginning of TFFLR superfusion, obtained from 11 neurons, are summarized in Fig. 4B. Figure 4C demonstrates a dose-response relationship for sEPSC frequency increase produced by TFFLR in a range of 0.01–5 μM; each of the data points was obtained from 4 to 11 neurons. Analysis based on the Hill equation showed that the effective concentration of TFFLR for half-maximal effect (EC50) is 0.32 μM with the Hill coefficient of 0.70.

A proteinase thrombin (0.134 National Institutes of Health units/ml), which is known to activate PAR-1 (Vergnolle et al. 2001), also increased the frequency of sEPSC without a change in its amplitude (Fig. 5A). This thrombin-mediated increase in sEPSC frequency was reduced in extent by PAR-1 antagonist YFLLRN (see Fig. 5B), indicating an involvement of PAR-1 but not PAR-3 and PAR-4 (see Vergnolle et al. 2001). Each of the results was obtained from three other neurons; Fig. 5C gives sEPSC frequency and amplitude, relative to those in the control, under the action of thrombin in the absence and presence of YFLLRN.

To elucidate whether the facilitatory action produced by PAR-1 activation is accompanied by an increase in neuronal activity, we next examined how TTX (0.5 μM) affects the actions of SFLLRN (1 μM) on spontaneous excitatory transmission. As seen in Fig. 6Aa, SFLLRN enhanced sEPSC frequency in the presence of TTX. Figure 6Ab gives sEPSC frequency and amplitude ~3.5 min after the beginning of SFLLRN superfusion, relative to control, in Krebs solution without and with TTX. TTX did not significantly affect the facilitatory action of SFLLRN on sEPSC frequency, indicating no involvement of TTX-sensitive neuronal activity increase in the SFLLRN action.

The SFLLRN-induced sEPSC frequency increase is thought to be due to an increase in intraterminal Ca2+ concentration, which may originate from Ca2+ in extracellular solution. To address this issue, we next examined the effect of SFLLRN (1 μM) on spontaneous excitatory transmission in a nominally Ca2+-free, high-Mg2+ (5 mM) Krebs solution. As reported previously (Yue et al. 2005), superfusing Ca2+-free Krebs solution reduced the frequency but not amplitude of sEPSC; under this condition SFLLRN did not affect sEPSC frequency (see Fig. 6Ba). A similar result was obtained from five other neurons; Fig. 6Bb gives sEPSC frequency and amplitude, relative to those in the control, just before and ~3.5 min after the beginning of SFLLRN superfusion.

The spontaneous release of L-glutamate onto SG neurons originates from the terminals of interneurons in the spinal dorsal horn and of primary-afferent neurons. To know which
types of neurons are involved in the PAR-1-mediated sEPSC frequency increase, we examined the effect of TFLLR (1 μM) superfused for 5 min on monosynaptically evoked Aδ- and C-fiber EPSC amplitudes in SG neurons. Stimulating the dorsal root with a strength of 23 mV (sufficient to recruit Aδ-fibers) elicited in some neurons monosynaptic EPSCs that displayed no failure and no change in latency when examined at 20 Hz (see Fig. 7Aa, left). CV values estimated from the latency of EPSC averaged to be 3.9 ± 0.3 m/s (range: 3.3– 4.7 m/s; n = 5); this was within the range of those of Aδ-fibers, obtained from experiments in DRG neurons, as reported previously (Ataka et al. 2000; Nakatsuka et al. 2000; also see Lao et al. 2004; Luo et al. 2002). Monosynaptic Aδ-fiber EPSCs evoked at 0.1 Hz had a mean amplitude of 179 ± 41 pA (range: 54–264 pA; n = 5; see Fig. 7Aa, right). On the other hand, stimuli with a strength >300 μA (enough to activate C-fibers) evoked in some neurons monosynaptic EPSCs which have no failures albeit a variety in the latency (see Fig. 7Ba, left). The monosynaptic EPSCs had an average CV of 0.30 ± 0.02 m/s (range: 0.24–0.34 m/s; n = 4), values comparable to those of C-fibers (Ataka et al. 2000; Nakatsuka et al. 2000; also see Lao et al. 2004; Luo et al. 2002). Monosynaptic C-fiber EPSCs evoked at 0.1 Hz had a mean amplitude of 78 ± 22 pA (range: 48–142 pA; n = 4; see Fig. 7Ba, right). As seen from Fig. 7, Ab and Bb, monosynaptic Aδ-fiber and C-fiber EPSC amplitudes were not affected by TFLLR; these results were confirmed in four and three other neurons, respectively. The peak amplitudes of monosynaptic Aδ-fiber and C-fiber EPSC around 3.5 min after the beginning of TFLLR superfusion were 102 ± 2% (n = 5; P > 0.05) and 96 ± 2% (n = 4; P > 0.05) of control, respectively.

All of the peptides examined did not alter holding currents at −70 mV, which is close to resting membrane potentials.

**DISCUSSION**

The present study demonstrated in SG neurons that PAR-1 ligand peptide SFLLRN increases sEPSC frequency and also a proportion of sEPSCs having a shorter inter-event interval.
without a change in sEPSC amplitude when compared with control. These results indicate a presynaptic facilitatory action of SFLLRN, i.e., an increase in the spontaneous release of L-glutamate from nerve terminals without a change in the sensitivity of non-NMDA receptors for L-glutamate. This presynaptic facilitatory effect was distinct from that at myenteric neuron synapses where the release of acetylcholine from nerve terminals was reduced by PAR ligands (Gao et al. 2002).

Although SFLLRN used here is an agonist peptide corresponding to a tethered ligand of human PAR-1, this ligand as well as PAR-1 agonist peptide TFLLRN (almost the same peptide as TFLRN used in our study) depolarizes membranes of guinea pig myenteric neurons (Gao et al. 2002), suggesting that the human PAR-1 agonist peptide also activates rodent PAR-1.

Although SFLLRN may act on PARs other than PAR-1, the SFLLRN-induced sEPSC frequency increase was reduced in extent by a PAR-1 antagonist peptide YFLLRNP and was mimicked by a selective PAR-1 agonist peptide TFLLR. PAR-2 and -4 ligand peptides (SLIGKV and GYPGQV, respectively) had minimally detectable influence on the frequency of sEPSC in SG neurons; the former result may be consistent with the observation that PAR-2 agonists had little effect on spontaneous glutamatergic transmission in a subtype of SG neurons in young rats (Alier et al. 2008). A PAR-1-activating proteinase thrombin (Vergnolle et al. 2001) also produced a similar increase in sEPSC frequency in a manner sensitive to YFLLRNP. Altogether, these data indicate an involvement of PAR-1 in the presynaptic action of SFLLRN.

All of PAR ligand peptides examined in the present study did not alter holding currents at −70 mV, although PAR-1, -2, and -4 ligand peptides have been reported to depolarize membranes of myenteric neurons (Gao et al. 2002; Linden et al. 2001).

FIG. 6. The sEPSC frequency increase produced by SFLLRN (1 μM) is resistant to TTX (0.5 μM) and is inhibited in a nominally Ca\(^{2+}\)-free Krebs solution. Aa: recordings of sEPSCs in the absence and presence of SFLLRN in Krebs solution containing TTX. Ab: sEPSC frequency (left) and amplitude (right) in the presence of SFLLRN (as measured for 30 s around 3.5 min after the beginning of its superfusion) without and with TTX, relative to those just before SFLLRN superfusion [7.1 ± 1.1 Hz, 13.5 ± 1.4 pA (n = 11) in the presence of TTX], where the data in the absence of TTX were adopted from Fig. 3C. Ba: recordings of sEPSCs in the absence and presence of SFLLRN in Ca\(^{2+}\)-free Krebs solution. Bb: sEPSC frequency (left) and amplitude (right) in Ca\(^{2+}\)-free Krebs solution without and with SFLLRN (as measured for 30 s around 3.5 min after the beginning of its superfusion), relative to those in normal Krebs solution (frequency: 7.7 ± 1.8 Hz, amplitude: 17.0 ± 3.0 pA; n = 6). V\(_{m}\) = −70 mV.

FIG. 7. TFLLR (1 μM) does not affect monosynaptic primary-afferent A\(\delta\)-and C-fiber EPSC amplitude. Aa: superimposition of 20 (left) or 6 traces (right) of the A\(\delta\)-fiber EPSCs; they were evoked at 20 or 0.1 Hz, respectively. Ba: average traces of 3 consecutive A\(\delta\)-fiber EPSCs (measured for 30 s; stimulated at 0.1 Hz) in the control (left) and around 3.5 min after the beginning of TFLLR superfusion (right). Bc: superimposition of 20 (left) or 6 traces (right) of the C-fiber EPSCs; they were evoked at 1 or 0.1 Hz, respectively. Bb: average traces of 3 consecutive C-fiber EPSCs (measured for 30 s; stimulated at 0.1 Hz) in the control (left) and around 3.5 min after the beginning of TFLLR superfusion (right). In each of A and B, the traces in a and b were obtained from the same neuron. V\(_{m}\) = −70 mV.
When compared in the efficacy of PAR-1 ligand peptides among preparations, EC50 value (0.32 μM) for TFLLRN in increasing sEPSC frequency in our study was close to that (0.358 μM) for SFLLRN in producing membrane depolarization in myenteric neurons (Gao et al. 2002) while being somewhat smaller than that (6.1 μM) for TFLLRN in evoking the release of l-glutamate from cortical astrocytes in culture (Lee et al. 2007). This difference in EC50 values may be due to the fact that the l-glutamate release from astrocytes is nonspecific one and is measured by using HEK 293 cells expressing GluR1 as a detector.

Although the spontaneous l-glutamate release onto SG neurons originates from the terminals of interneurons and primary-afferent neurons, no effect of PAR-1 agonist on monosynaptic Aδ- and C-fiber-evoked EPSC amplitudes indicates that this agonist may act on PAR-1 existing in the terminals of glutamatergic interneurons in the spinal dorsal horn. This result is consistent with the presence of mRNAs for PAR-1 in the rat spinal cord (Kawabata et al. 2001; see also Weinstein et al. 1995) and of PAR-1-like immunoreactivity in the mouse spinal dorsal horn (Narita et al. 2005) and also with the observation that sEPSCs in the SG are predominantly mediated by the spontaneous release of l-glutamate from interneuron terminals rather than from primary-afferent terminals (Yang and Li 2001).

The presynaptic action of SFLLRN is resistant to TTX and thus not due to an increase in the activities of TTX-sensitive neurons. It is possible that this action is mediated by a chemical substance released from nonneuronal cells such as astrocytes as a result of an action of SFLLRN, because it is known that astrocytes in the CNS express PARs (for example, see Lee et al. 2007; Wang et al. 2002). Koetzner et al. (2004) have reported that the intrathecal administration of thrombin results in the release of prostaglandin E2 (PGE2), possibly from nonneuronal tissues, in the rat spinal cord. It is, however, unlikely that PGE2 is involved in the presynaptic action of SFLLRN in the SG because PGE2 does not change sEPSC frequency in adult rat SG neurons (Baba et al. 2001).

The activation of PARs in cultured astrocytes is known to increase intracellular Ca2+ concentrations (Wang et al. 2002); Lee et al. (2007) have demonstrated that an increase in intracellular Ca2+ concentration by PAR-1 activation in cultured cortical astrocytes or acutely dissociated hippocampal astrocytes results in the release of l-glutamate from there. The spontaneous release of l-glutamate from nerve terminals in the SG occurs in a manner dependent on extracellular Ca2+ (Hori et al. 1992; Yue et al. 2005). Thus it is likely that the PAR-1-mediated increase in the spontaneous release of l-glutamate in the SG is due to intracellular Ca2+ concentration increase in nerve terminals; this may be due to PAR-1 activation in not only nerve terminals but also nonneuronal cells, the latter of which activations results in the release of a substance acting on SG nerve terminals (see the preceding text). This increase in intracellular Ca2+ concentration appears to be due to Ca2+ influx from extracellular solution, because the SFLLRN-induced sEPSC frequency increase is not seen in a nominally Ca2+-free solution. PAR-1 is known to couple with heterotrimeric G proteins at the plasma membrane, resulting in the commencement of a variety of signal transductions (for review, see Noorbakhsh et al. 2003; Ossovskaya and Bunnett 2004). It remains to be examined what kinds of signal transductions are involved in the PAR-1 effect in SG neurons.

Since it has been reported that PAR-1 and -2 activation in DRG neurons results in the release of substance-P (SP) from their peripheral terminals (de Garavilla et al. 2001; Steinhoff et al. 2000; for review, see Ceccac and Vergnolle 2005), this release may have also occurred in the central terminals of the neurons, leading to the production of a SP response in SG neurons. However, this was not the case in the present study, because PAR-1 and -2 ligand peptides did not alter holding currents in SG neurons. This may be due to an unresponsiveness of SG neurons to SP (see Yang et al. 2000).

**Physiological significance of PAR-1 activation in the SG**

The sEPSC frequency increase produced by PAR-1 activation is an action opposite to that of endogenous neuropeptides such as endorphinins (Fujita and Kumamoto 2006; Wu et al. 2003) and nociceptin (Liebel et al. 1997; Luo et al. 2002) and of adenosine (Lao et al. 2001; Li and Perl 1994), all of which are thought to act as analgesic at the spinal cord level (for review, see Fürst 1999). Considering that sensory processing within the SG plays a pivotal role in regulating nociceptive transmission (Willis and Coggeshall 1991), the present finding suggests that PAR-1-mediated glutamatergic transmission enhancement in the SG may play a role in the modulation of nociception. Consistent with this idea, it has been reported that the intrathecal administration of thrombin and also SFLLRN produces thermal hyperalgesia in rodents (Koetzner et al. 2004; Narita et al. 2005). On the other hand, Fang et al. (2003) have reported that a transient mechanical hyperalgesia induced by intrathecally administered NMDA in mice is inhibited by thrombin and also TFLLRN, possibly through a pathway involving endothelin type A receptors activated by endothelin-1 mobilized from astrocytes, i.e., thrombin exhibits an antinociceptive rather than a hyperalgesic effect. A role of PAR-1 in the regulation of nociceptive transmission at the spinal cord level remains to be further examined. It is of interest to note that the intraplantar administration of PAR-1 ligand peptides attenuates carrageenan-induced hyperalgesia in rats (Asfaha et al. 2002; Kawabata et al. 2002). PAR-1 may play a distinct role between the CNS and PNS in modulating nociceptive transmission.

Although PAR-1 activating proteinase thrombin produces an action similar to that of PAR-1 ligand peptides in the SG, thrombin cannot penetrate the blood-brain barrier while mRNAs of its precursor prothrombin are detected in the DRG and CNS (Dihanich et al. 1991) and Factor Xa (a protein that converts prothrombin to thrombin)-like immunoreactivity is found in the CNS (Yamada and Nagai 1996). The integrity of the barrier is known to be compromised by pathological states such as hemorrhagic stroke (Gingrich and Traynelis 2000). It remains to be addressed under what conditions and by what kinds of endogenous proteases PAR-1 in the SG is activated.

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