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REDUCTION OF GLYCINE RECEPTOR-MEDIATED MINIATURE INHIBITORY POSTSYNAPTIC CURRENTS IN RAT SPINAL LAMINA I NEURONS AFTER PERIPHERAL INFLAMMATION

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Abstract—Peripheral inflammation may induce long-lasting sensitization in the central nociceptive system. Neurons in lamina I of the spinal dorsal horn play a pivotal role in the integration and relay of pain-related information. In rats we studied whether changes in passive and active membrane properties and/or alteration of glycine receptor-mediated inhibitory control of spinal lamina I neurons may contribute to central sensitization in a model of peripheral long-lasting inflammation (complete Freund's adjuvant, hindpaw). Spontaneously occurring glycine receptor-mediated miniature inhibitory postsynaptic currents (GlyR-mediated mIPSCs) were recorded in lumbar spinal lamina I neurons. Miniature IPSC rise, decay kinetics and mean GlyR-mediated mIPSC amplitude were not affected by peripheral inflammation. The mean frequency of GlyR-mediated mIPSCs of lamina I neurons ipsilateral to the inflamed hindpaw was, however, significantly reduced by peripheral inflammation when compared with neurons from noninflamed animals. Principal passive and active membrane properties and firing patterns of spinal lamina I neurons were not changed by inflammation.

These results indicate that long-lasting peripheral inflammation leads to a reduced glycinergic inhibitory control of spinal lamina I neurons by a presynaptic mechanism.

Key words: nociception, pain, central sensitization, inhibitory control, allodynia, complete Freund's adjuvant.

Persistent peripheral inflammation is typically associated with a pain sensation in response to normally non-painful stimuli (allodynia) and/or abnormally intense pain elicited by noxious stimuli (hyperalgesia). This pain hypersensitivity can be attributed both to a sensitization of nociceptors in the vicinity of the damaged tissue (peripheral sensitization; Levine and Taiwo, 1994) and to a sensitization of nociceptive neurons in the CNS (central sensitization; Woolf, 1983; Willis, 2001; Ikeda et al., 2003).

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Abbreviations: bicuculline, bicuculline methiodide; CFA, complete Freund's adjuvant; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; D-AP5, D-2-amino-5-phosphonovaleric acid; GlyR-mediated mIPSCs, glycine receptor-mediated miniature inhibitory postsynaptic currents; TTX, tetrodotoxin.

Neurons in lamina I of the spinal cord dorsal horn are of great importance for the integration and transmission of nociceptive information. They receive nociceptive input from small caliber myelinated (Aδ) and unmyelinated (C) primary afferents and lamina I functions as the main output source of the superficial spinal dorsal horn to supraspinal sites (Craig, 2000). Recently, it was shown that a subset of spinal lamina I neurons plays a pivotal role in the generation and maintenance of thermal hyperalgesia and mechanical allodynia in persistent neuropathic and inflammatory pain states (Nichols et al., 1999).

The cellular mechanisms of central sensitization are not completely understood. In particular it is not known whether cell hyperexcitability due to altered membrane properties or altered discharge properties of spinal lamina I neurons may contribute to central sensitization. Thus, we studied these questions in a rat model of long-lasting peripheral inflammation induced by injection of complete Freund's adjuvant (CFA) into one hindpaw.

Glycine functions as a fast inhibitory neurotransmitter in the superficial spinal dorsal horn (van den Pol and Gorcs, 1988; Todd, 1990) modulating spinal lamina I neuron excitability by tonic inhibition (Chéry and De Koninck, 1999). Administration of the glycine receptor antagonist strychnine increases input from low-threshold mechanoreceptors and leads to frank pain behavior in awake rats (Yokota et al., 1979; Yaksh, 1989) whereas iontophoretic administration of glycine reduces spontaneous activity in spinothalamic tract cells and inhibits their responses evoked by stimulation of their receptive fields (Zieglgänsberger and Herz, 1971; Willcockson et al., 1984).

Transmission of afferent nociceptive information strongly depends upon the discharge properties of higher order neurons. Recently, we have described a lamina-specific distribution of firing patterns among spinal dorsal horn neurons (Ruscheweyh and Sandkühler, 2002).

Here, we studied whether peripheral long-lasting inflammation affects glycinergic inhibitory control and/or the distribution of firing patterns among spinal lamina I neurons.

EXPERIMENTAL PROCEDURES

Preparation of animals and spinal cord slices

Long-lasting inflammation was induced by injection of 100–150 μl CFA (Sigma, Deisenhofen, Germany) into the plantar surface of one hindpaw of 16- to 26-day-old Sprague–Dawley rats of both sexes under ether anesthesia. Edema and erythema of the hindpaw as well as behavioral changes (limp, paw elevation, licking,

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shaking) were assessed for the first hour after injection and immediately before preparation of spinal cord slices 72 h after injection, because the resulting long-lasting pain syndrome is peaking at that time point (Iadarola et al., 1988).

Lumbar spinal cord was exposed under deep ether anesthesia by laminectomy. Lumbar spinal cord segments L4 and L5 were excised and cut into transverse slices of 400–500 μm thickness using a vibrating microslicer (DTK-1000; Dosaka EM, Kyoto, Japan). The spinal dorsal horn contralateral to the inflamed hindpaw was marked by a small cut for later identification. The slices were stored in an incubation solution equilibrated with carbogen (5% CO_2 in 95% O_2) and containing (in mM): NaCl, 95; KCl, 1.8; KH_2PO_4, 1.2; $CaCl_2$, 0.5; $MgSO_4$, 7; $NaHCO_3$, 26; glucose, 15; sucrose, 50; pH was 7.4, measured osmolality 310 to approximately 320 mosmol/kg. A single slice was then transferred to a recording chamber (volume 1.0 ml) and was continuously superfused at a rate of 3–4 ml/min with a recording solution (aerated with carbogen), which was similar to the incubation solution except for (in mM): NaCl, 127; $CaCl_2$, 2.4; $MgSO_4$, 1.3; sucrose 0. All experiments were performed at room temperature (20–25 °C).

Labeling of spino-parabrachial projection neurons

Young rats were anesthetized with a mixture of ketamine and xylazine and placed in a stereotaxic apparatus. A hole was drilled through the skull in order to allow insertion of a 500-nl Hamilton syringe needle. The animals received a single injection of 200 nl of 2.5% 1,1-Didodecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiIC12; Molecular Probes, Eugene, OR, USA) into the right parabrachial area according to the atlas of Paxinos and Watson (1982). After a 3-day survival period spinal cord slices were obtained as described above. All animal experiments were performed in accordance with European Communities Council directives (86/609/EEC) and were approved by the Austrian Federal Ministry for Education, Science and Culture. All efforts were made to minimize the required number of animals and their suffering.

Recording techniques and data acquisition

Neurons in lamina I of the spinal dorsal horn showing a more mediolateral orientation of their proximal axon/dendrites in contrast to the smaller lamina II neurons with a mainly dorsoventral orientation of proximal axon/dendrites were visualized with Dodt-infrared optics using a ×40, 0.80 water-immersion objective on an Olympus BX50WI upright microscope (Olympus, Japan) equipped with a video camera system (PCO, Kelheim, Germany). To detect labeled projecting neurons and non-labeled cells, slices were illuminated with a monochromator (TILL photonics, Gräfelfing, Germany).

Standard patch-clamp recordings were made in the whole-cell configuration. Patch pipettes were pulled from borosilicate glass capillaries (GC 150–15; Clark Electromedical Instruments, UK) on a horizontal puller (P-87; Sutter Instruments, Novato, CA, USA). For measuring membrane properties and firing patterns, pipettes were filled with a solution composed of (in mM): potassium gluconate, 120; KCl, 20; $MgCl_2$, 2; HEPES, 10; EGTA, 0.5; Na_2ATP, 2; NaGTP, 0.5; pH 7.28 adjusted with KOH; osmolality approximately 300 mosmol/kg, resulting in a tip resistance between 3 and 6 MΩ. Firing patterns were obtained in response to 1 s depolarizing current pulses of different intensities (25–250 pA, 25 pA steps). For recording of glycine receptor-mediated miniature inhibitory postsynaptic currents (GlyR-mediated mIPSCs), tetrodotoxin (TTX, 0.5 μM; Alexis, Grünstadt, Germany), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM; Alexis), D-2-amino-5-phosphonovaleric acid (D-AP5, 50 μM; Alexis) and bicuculline methiodide (bicuculline, 10 μM; Sigma) were added to the bathing solution. For these experiments the pipette solution contained (in mM): CsCl, 140; $MgCl_2$, 2; $CaCl_2$, 1; HEPES, 10; EGTA, 5; Na_2ATP, 2; NaGTP, 0.5; pH 7.28 adjusted with CsOH (pipette resistances 2–5 MΩ). Recordings were performed in voltage-clamp mode at a holding potential of −55 mV. In some experiments 4 μM strychnine (Sigma) was applied to block GlyR-mediated mIPSCs. Recordings were obtained from visually identified lamina I neurons ipsilateral to the inflamed hindpaw using an Axopatch 200B patch-clamp amplifier and pClamp 8 software (Axon Instruments, Union City, CA, USA). Data were low-pass filtered at 5 kHz (bessel filter) and digitized at 10 kHz with a Digidata 1320 A/D converter (Axon Instruments). The access resistance was monitored throughout each experiment. Only recordings with an access resistance less than 35 MΩ, which were stable throughout the experiment were considered acceptable for analysis of GlyR-mediated mIPSCs. At the end of each experiment the position of the tip of the recording electrode was visualized through a 4× objective to verify the recording site in lamina I. Lamina I was identified as a small strip on the edge of the dorsal white matter dorsally to the translucent band of lamina II. Recordings from outside lamina I were excluded from data analysis. Controls were obtained from neurons of untreated rats.

Data analysis

The software program pClamp 8 was used for off-line data analysis. Firing patterns were assessed by analyzing responses to all current intensities applied. Neurons which did not show definite firing patterns were excluded from further comparison. For GlyR-mediated mIPSC analysis the Mini Analysis Program (Synaptosoft, Decatur, USA) was used. The software-detected events were re-evaluated by visual inspection; any noise which spuriously met the trigger specifications was rejected. GlyR-mediated mIPSCs were only included in decay phase analysis if: 1) the baseline was stable before and after the event and 2) the event did not show multiple peaks. Decay times were measured between 90 and [1/e] % of peak amplitude of the mIPSC. Rise times were determined between 10 and 90% of the peak amplitude.

The critical value for statistical significance was set at $P<0.05$. The non parametric Mann-Whitney rank test was used for statistical comparisons. All data are expressed as means±1 S.E.M.

RESULTS

After injection of CFA into one hindpaw rats showed limp, paw elevation, licking and shaking of the inflamed hindpaw within the first hour after waking up from ether anesthesia. All rats developed erythema and paw swelling lasting until the slice preparation of the spinal cord 72 h after the injection (data not shown).

Principal passive and active intrinsic membrane properties of spinal lamina I neurons were not affected by CFA-induced peripheral long-lasting inflammation (Table 1).

In spinal lamina I neurons of untreated animals five different firing patterns could be distinguished (Fig. 1A). Tonic firing neurons discharged regularly with little spike attenuation and frequency adaptation in response to 1 s depolarizing current pulses (Fig. 1A, first column). Delayed firing neurons showed a characteristic slow ramp depolarization to subthreshold current pulses and discharged irregularly after a delayed first action potential in response to higher current intensities (Fig. 1A, second column). Initial bursting neurons discharged only a short burst of action potentials with marked spike attenuation and frequency adaptation at the onset of the depolariz-

Table 1. Passive and active membrane properties of spinal lamina I neurons are not significantly changed during course of CFA-induced peripheral inflammation[a]

	Control	CFA
Resting membrane potential (mV)	−57.4±1.3 (*n*=21)	−61.1±1.5 (*n*=28)
Membrane resistance (MΩ)	583.0±50.1 (*n*=23)	605.0±41.7 (*n*=21)
Membrane capacitance (pF)	38.3±3.1 (*n*=23)	38.7±2.6 (*n*=21)
Action potential threshold (mV)	−35.8±1.2 (*n*=21)	−37.6±0.8 (*n*=28)

[a] Values are means±S.E.M.

ing current pulses (Fig. 1A, third column). Single spiking neurons fired one action potential at the beginning of the depolarization (Fig. 1A, fourth column). Phasic bursting neurons discharged bursts of action potentials irregularly at different time intervals and of varying length in response to 1 s depolarizing current injections (Fig. 1A, fifth column). Tonic firing neurons were found most frequently in lamina I (42.9%; Fig. 1B; *n*=21), followed by delayed firing neurons (23.8%), initial bursting and single spiking neurons (both 9.5%) and phasic bursting neurons (4.8%). Some cells showed no definite firing patterns (9.5%). The same types of firing patterns in a similar frequency distribution were also found in CFA-injected rats (Fig. 1C; *n*=28). Neither the resting membrane potential nor the action potential threshold were altered by CFA-induced peripheral inflammation in subsets of lamina I neurons classified by the firing patterns described above (data not shown).

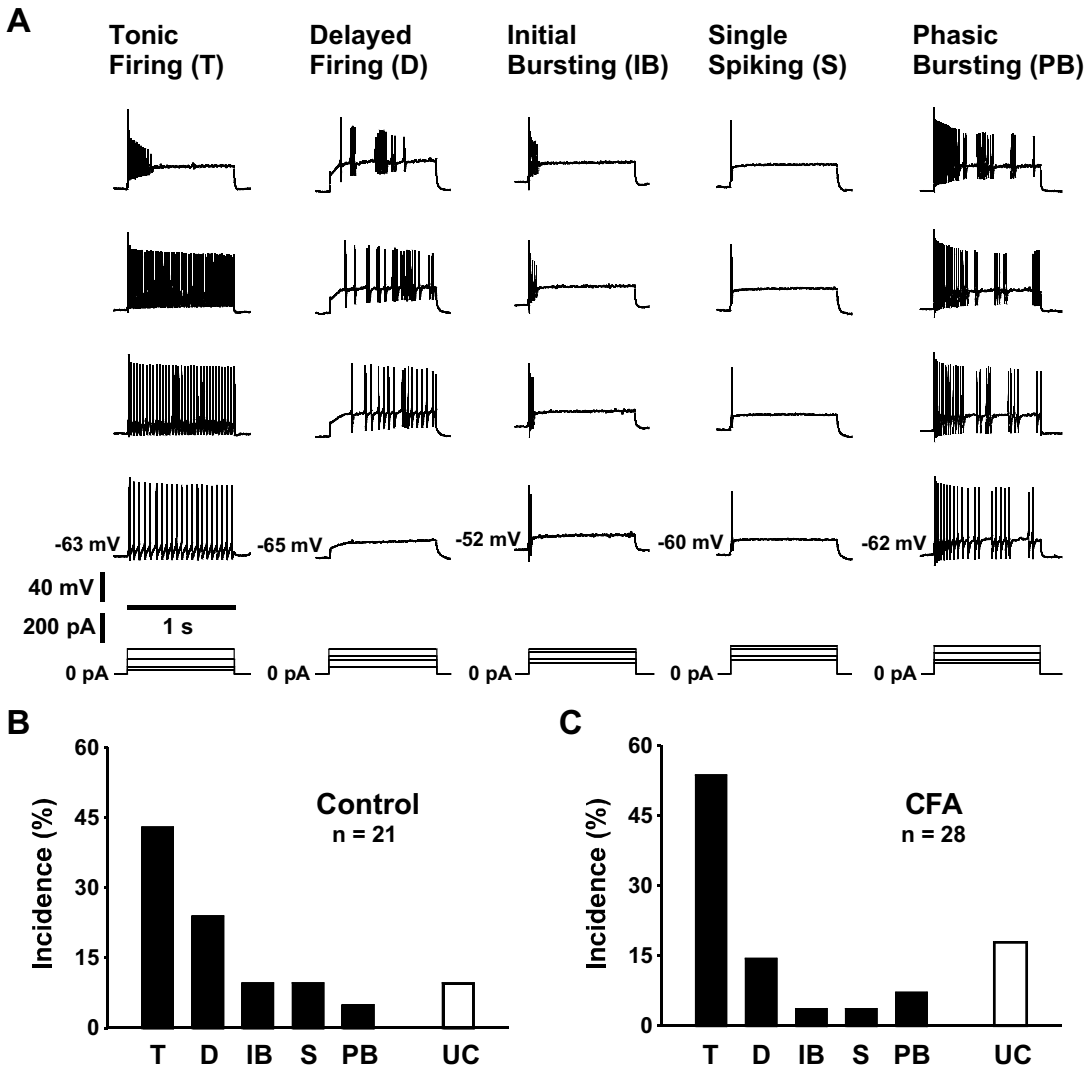

Fig. 1. Peripheral inflammation does not change firing pattern distribution of spinal lamina I neurons. (A) Five different firing patterns were recorded in spinal lamina I neurons in response to 1 s depolarizing current pulses (25–250 pA, 25 pA steps) applied from resting membrane potential in current clamp mode. Selective traces from representative neurons are shown. Bottom traces in the five columns show current injections (superimposed). (B, C) The firing pattern distribution shows no qualitative differences between ensembles of neurons from control animals (B) compared with those from animals with a CFA-induced long-lasting peripheral inflammation (C). UC, unclassified.

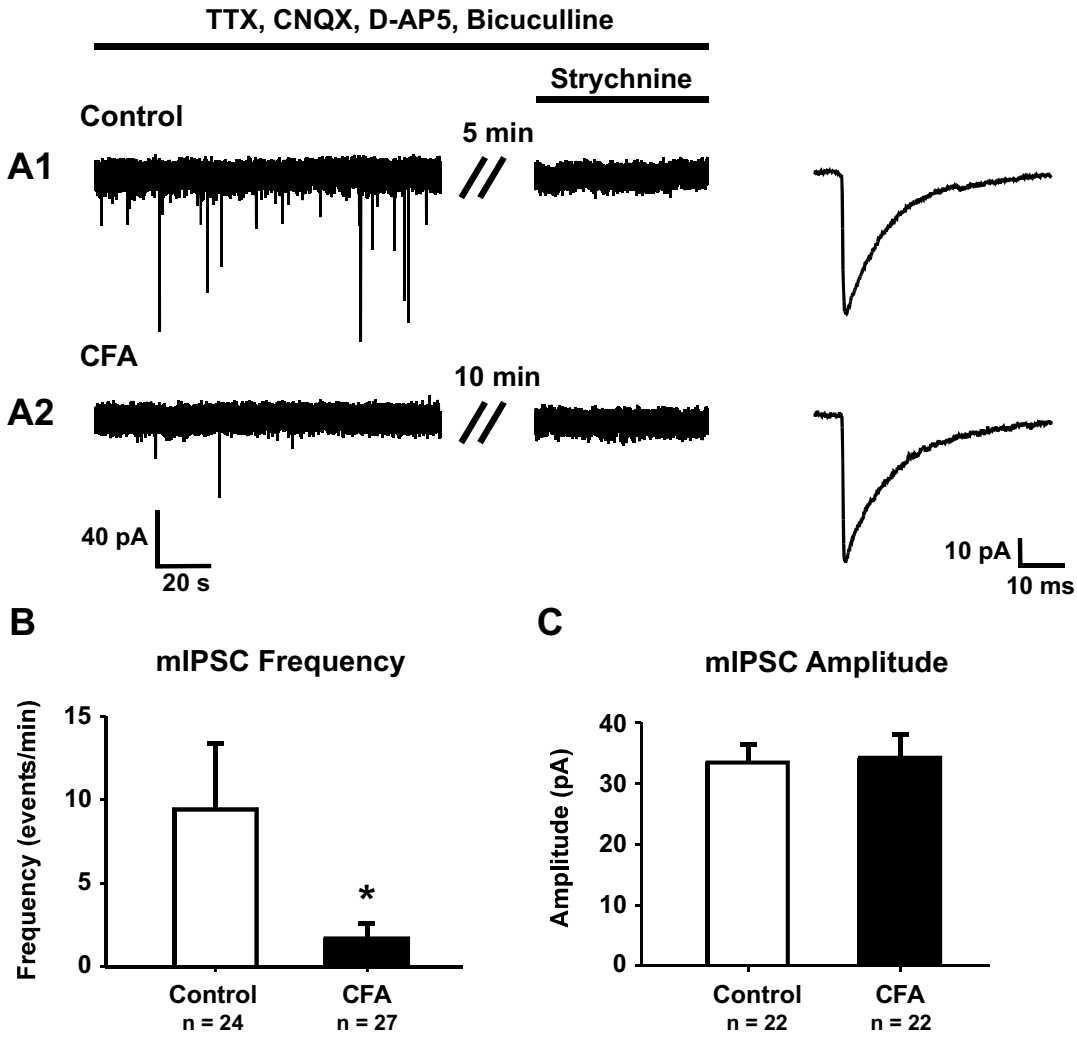

Fig. 2. Glycinergic mIPSCs are reduced in frequency but are neither altered in amplitude nor in kinetics after peripheral inflammation. (A) Representative traces of GlyR-mediated mIPSCs from a control spinal lamina I neuron (A1) and from a spinal lamina I neuron from an animal with CFA-induced peripheral inflammation (A2). Current recordings were made in presence of TTX (0.5 μM), CNQX (10 μM), D-AP5 (50 μM) and bicuculline (10 μM) in voltage-clamp mode (V_{Hold} = −55 mV). Bath application of strychnine (4 μM) abolished mPSCs which are then GlyR-mediated. Averages of glycinergic mIPSCs of traces in A1 and A2 are shown on the right (alignment of mIPSCs by 50% rise time). (Neuron in A1) frequency = 14.7 events/min, mean amplitude = 41 pA, rise time = 0.77 ms, decay time = 7.7 ms, GlyR-mediated mIPSC average: n = 43; (Neuron in A2) frequency = 1.7 events/min, mean amplitude = 56 pA, rise time = 0.54 ms, decay time = 6.0 ms, GlyR-mediated mIPSC average: n = 32. (B) Mean GlyR-mediated mIPSC frequency of spinal lamina I neurons is significantly lower during course of 72 h lasting CFA-induced peripheral inflammation. (C) Mean GlyR-mediated mIPSC amplitude shows no significant differences between control cells compared with cells from animals with a peripheral long-lasting inflammation.

In the presence of TTX (0.5 μM), CNQX (10 μM), D-AP5 (50 μM) and bicuculline (10 μM) GlyR-mediated mIPSCs were recorded in spinal lamina I neurons. These mIPSCs were blocked by bath application of 4 μM strychnine (Fig. 2A). The average rise time of GlyR-mediated mIPSCs in control lamina I neurons was 0.65±0.06 ms (n = 17), not significantly different from the rise time of glycinergic mIPSCs in lamina I neurons from animals with CFA-induced peripheral chronic inflammation (0.56±0.04 ms, n = 13, P > 0.2). Similarly, the average decay time of GlyR-mediated mIPSCs was 7.7±0.7 ms in controls (n = 13), comparable to the decay time of glycinergic mIPSCs in neurons from rats with long-lasting inflammation (6.4±0.8 ms; n = 11; P > 0.2). The mean frequency of GlyR-mediated mIPSCs in the control ensemble of spinal lamina I neurons was 9.4±3.9 events/min (n = 24), which was significantly reduced to 1.7±0.9 events/min (n = 27, P < 0.05; Fig. 2B) during course of long-lasting inflammation, ipsilateral to the inflamed hindpaw. In contrast, the mean GlyR-mediated mIPSC amplitude was not significantly different between control lamina I neurons (33.3±2.9 pA; n = 22) compared with cells from animals with a peripheral inflammation (34.3±3.7 pA; n = 22; P > 0.5; Fig. 2C). The access resistance in these experiments was not significantly different between the two cell populations (12.5±0.7 MΩ vs. 15.1±1.3 MΩ; control vs. CFA; P > 0.1) and neither were passive membrane properties

(membrane resistance and membrane capacitance; data not shown).

To test if there is a difference between unidentified lamina I neurons and projecting neurons, we measured the effect of CFA-induced peripheral inflammation on the glycinergic input of spino-parabrachial neurons. The incidence of glycinergic mIPSCs was very low, both in nine neurons from control animals and in seven neurons from animals with an inflamed hindpaw (data not shown).

DISCUSSION

The main finding of the present study is a reduction of glycinergic inhibition of spinal lamina I neurons following CFA-induced peripheral inflammation of 72 h duration. In contrast, neither principal passive and active membrane properties nor distribution of firing patterns among spinal lamina I neurons were affected. Similarly, the resting membrane potential remains stable during CFA-induced peripheral inflammation of 48 h duration in lamina II neurons (Baba et al., 1999). Thus, central sensitization during long-lasting peripheral inflammation involves loss of glycinergic inhibition but does not seem to be based on changes of passive and active membrane properties of superficial spinal dorsal horn neurons.

The distribution of discharge patterns identified in our previous study for lamina I neurons (Ruscheweyh and Sandkühler, 2002) were confirmed in the present study, both, in naive animals and in animals with an inflammation of the hind-paw. This suggests that discharge pattern is a robust feature of lamina I neurons that does not change by persistent inflammation. In neurons, projecting to the parabrachial area of the brainstem, we found a specific firing pattern in 72% of the cells, classified as gap firing because of a delay between the first and the following action potentials, elicited by depolarization (Ikeda et al., 2001). Since this firing pattern was rare in unidentified neurons (15%), it is likely that most of the neurons, showing a reduced glycinergic inhibition after CFA-induced inflammation did not project to the parabrachial area.

Little is known so far about modifications of the glycinergic inhibitory system in the spinal dorsal horn after peripheral nerve or tissue injury. After unilateral sciatic nerve constriction injury glycine receptor staining in spinal Rexed laminae II–IV is shown to decrease bilaterally (Simpson and Huang, 1998). Our results show for the first time that a localized long-lasting peripheral CFA-induced inflammation leads to a reduced glycinergic mIPSC frequency in spinal lamina I neurons whereas the GlyR-mediated mIPSC rise time, decay time and amplitude remain stable. These findings argue in favor of a presynaptic mechanism. In the sacral dorsal commissural nucleus in the spinal cord activation of metabotropic glutamate receptors on presynaptic glycinergic boutons inhibits glycinergic mIPSC frequency (Katsurabayashi et al., 2001). Recently, the neuropeptide nocistatin has been shown to selectively reduce neurotransmitter release from inhibitory interneurons in the rat spinal cord dorsal horn by a presynaptic mechanism (Zeilhofer et al., 2000). Thus, glutamate and nocistatin may have a role as an endogenous inhibitor of glycinergic neurotransmission underlying the frequency reduction of glycinergic mIPSCs in spinal lamina I neurons. The cellular mechanisms altering glycinergic inhibitory neurotransmission in the spinal dorsal horn in models of long-lasting peripheral inflammation have not yet been studied in detail. Cell death of glycinergic interneurons results in a decreased number of synapses between glycinergic inhibitory interneurons and spinal lamina I neurons. Prolonged perforant path stimulation is known to induce both necrotic and apoptotic neuronal death in the hippocampus (Sloviter et al., 1996). Glycinergic inhibitory interneurons in the spinal dorsal horn presumably receive a major monosynaptic input from myelinated low threshold and unmyelinated primary afferents (Todd, 1990). Primary afferent A-fiber activity can cause neuronal cell death in laminae I–III of the spinal dorsal horn with an extent depending on whether intact or injured fibers are activated (Coggeshall et al., 2001). Glycine-containing inhibitory interneurons in the spinal cord seem to be especially vulnerable when excessively stimulated by glutamatergic receptor activation (Kwak and Nakamura, 1995). Recently, Moore at al. (2002) have found apoptotic neuronal profiles in laminae I and II of the spinal cord after spared nerve injury. Thus, excessive glutamate release in the superficial spinal dorsal horn during course of long-lasting inflammatory peripheral tissue damage could be sufficient to induce necrotic and/or apoptotic cell death of glycinergic interneurons resulting in a loss of glycinergic inhibition of spinal lamina I neurons.

Prostaglandins are well known as mediators of inflammation and pain (Levine and Taiwo, 1994) and cyclooxygenase-2 upregulation in the spinal cord is reported to occur both during the acute and chronic phase in CFA-induced peripheral inflammation (Beiche et al., 1998). Recently, it has been shown that prostaglandin E_2 selectively blocks inhibitory glycinergic neurotransmission in spinal lamina II neurons via a postsynaptic mechanism (Ahmadi et al., 2002). Inhibition of spinothalamic tract neurons by iontophoretic application of glycine is diminished during central sensitization induced by intradermal injection of capsaicin (Lin et al., 1996). Taken together, the present and previous results suggest that both pre- and postsynaptic modifications of inhibitory glycinergic neurotransmission in the superficial dorsal horn occur during central sensitization, probably depending upon the kind and duration of peripheral tissue damage.

Lamina I of the spinal cord dorsal horn receives descending modulation from multiple sites in the brainstem (Craig, 2000). Periaqueductal gray and nucleus raphe magnus stimulation-induced inhibition of nociceptive dorsal horn neurons is associated with an increase in the release of various neurotransmitters in spinal dorsal horn one of which is glycine (Sorkin et al., 1993; Cui et al., 1999). Recently, Baba et al. (2000) found that glycinergic synaptic neurotransmission in the substantia gelatinosa is enhanced presynaptically via a_1-adrenergic receptors. Thus, loss of glycinergic inhibition of spinal lamina I neurons after long-lasting peripheral inflammation may repre-

sent reduced segmental and descending inhibitory modulation.

Inhibitory control of spinal lamina I projection neurons is crucial for defining the output of lamina I to higher brain sites which integrate noxious information. Glycinergic synapses are relatively infrequent in the superficial dorsal horn compared with the remainder of the spinal cord gray matter. However, Puskár et al. (2001) have identified a population of large lamina I neurons with high levels of the glycine receptor-associated protein gephyrin most of which were retrogradely labeled from the parabrachial area. Here, we did not find any significant glycinergic input for lamina I spino-parabrachial neurons. The reasons for this discrepancy are not known at present. The frequency of GlyR-mediated mIPSC differed widely among lamina I neurons suggesting a non-uniform glycinergic input to different subpopulations of lamina I neurons. Thus, plasticity of the glycinergic inhibitory system in the superficial dorsal horn after peripheral inflammation may be of differential importance for lamina I neurons.

The reduced glycinergic inhibitory input to some lamina I neurons of the spinal dorsal horn following peripheral CFA-induced inflammation is likely of relevance for the induction and maintenance of allodynia (Sherman and Loomis, 1994; Sorkin and Puig, 1996). Using the *in vivo* patch-clamp technique, Narikawa et al. (2000) confirmed the importance of glycinergic neurotransmission in modulating nociceptive information in the spinal dorsal horn in showing that innoxious mechanical stimuli evoked IPSCs in 80% of substantia gelatinosa neurons most of which were mediated by glycine receptors. Sherman et al. (1997) have reported that after intrathecal administration of strychnine all nociceptive-specific neurons in the medial thalamus become responsive to normally innocuous tactile stimuli. These cellular changes parallel the onset of allodynia as seen by cardiovascular and motor responses to innocuous tactile stimuli. Thus, the present and previous results suggest that loss of glycinergic inhibition of spinal lamina I neurons may be an important mechanism in the generation and/or maintenance of allodynia in persistent peripheral inflammation.

CONCLUSIONS

CFA-induced peripheral inflammation leads to a reduction of glycinergic inhibitory control of spinal lamina I neurons. This may both contribute to and facilitate induction and maintenance of central sensitization by increasing the excitability of lamina I neurons leading to tactile allodynia.

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