Glial Nitric Oxide-Mediated Long-Term Presynaptic Facilitation Revealed by Optical Imaging in Rat Spinal Dorsal Horn

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We investigated a presynaptic form of long-term potentiation (LTP) in horizontal slices of the rat spinal cord by visualizing presynaptic and postsynaptic excitation with a voltage-sensitive dye. To record presynaptic excitation, we stained primary afferent fibers anterogradely from the dorsal root. A single-pulse test stimulation of C fiber-activating strength to the dorsal root elicited action potential (AP)-like or compound AP-like optical signals throughout the superficial dorsal horn. After conditioning (240 pulses at 2 Hz for 2 min), the presynaptic excitation was augmented. Furthermore, new excitation was elicited in the areas that were silent before conditioning. For postsynaptic recording, projection neurons in spinal lamina I were stained retrogradely from the periaqueductal gray in the brain stem. The test stimulation elicited AP-like or EPSP-like optical signals in the stained neurons. After conditioning, the EPSP-like responses were augmented, and previously silent neurons were converted to active ones. Results obtained with a nitric oxide (NO) donor, NO synthase inhibitors, metabotropic glutamate receptor (mGluR) agonist and mGluR1 antagonist, and a glial metabolism inhibitor suggest that after conditioning, presynaptic excitation is facilitated by NO released from glial cells via the activation of mGluR1. The results also indicate the possible presence of additional presynaptic and postsynaptic mechanism(s) for the LTP induction. Activity-dependent LTP of nociceptive afferent synaptic transmission in the spinal cord is believed to underlie central sensitization after inflammation or nerve injury. This glial NO-mediated control of presynaptic excitation may contribute to the induction at least in part.

Key words: long-term potentiation; LTP; hyperalgesia; optical imaging; spinal dorsal horn; nitric oxide; glia

Introduction

Primary afferent synapses in the superficial dorsal horn of the spinal cord undergo activity-dependent long-term potentiation (LTP). Synaptic transmission of unmyelinated C-afferent fibers is facilitated by conditioning electrical stimulation (Randić et al., 1993; Ikeda et al., 2000, 2003) and by natural noxious stimulation or nerve injury (Sandkühler and Liu, 1998; Sandkühler, 2000). Such central sensitization is thought to play important roles in hyperalgesia, an increased sensitivity to noxious stimuli, after peripheral inflammation or tissue injury (Treede et al., 1992; Woolf, 1994; Randić, 1996; Sandkühler, 1996; Wolpaw and Tենnissen, 2001). A recent study reports that LTP at C-afferent synapses on a group of projection neurons (PNs) in the dorsal lamina I is induced through a postsynaptic mechanism involving NMDA receptors (Ikeda et al., 2003). The projection neurons, sending axons to the medial parabrachial area, are believed to convey noxious information to the supraspinal regions. How-

ever, evidence indicates that LTP with different induction mechanism(s) is also present in the superficial dorsal horn. LTP at C-afferent synapses on lamina I neurons projecting to the periaqueductal gray matter (PAG) requires a different conditioning stimulation (CS) (Sandkühler and Ikeda, 2003). Activation of group I metabotropic glutamate receptors (mGluRs) facilitates afferent synaptic transmission (Zhong et al., 2000) and enhances glutamate release in the superficial dorsal horn (Park et al., 2004).

It is known that in central synapses, including those of spinal neurons, the conversion of previously silent synapses into active ones also underlies LTP (Liao et al., 1995; Li and Zhuo, 1998; Malenka and Nicoll, 1999; Frerking, 2001; Kim et al., 2003; Luscher and Isaac, 2003; Malinow, 2003; Voronin and Cherubini, 2003), in addition to increased efficacy of pre-existing synaptic transmission. Thus, we investigated whether silent synapses contribute to the LTP of C-afferent synapses and examined the induction mechanism, especially that at presynaptic sites.

In this study, we used optical methods (Tanifuji et al., 1994; Ikeda et al., 1998), because several hours of noninvasive recording from both presynaptic and postsynaptic sites could be achieved. The excitation of individual projection neurons in the superficial dorsal horn in spinal cord slices was visualized by retrograde staining with a voltage-sensitive dye, and excitation of presynaptic axons and terminals was visualized by anterograde staining from the dorsal root. We report that, after conditioning,

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excitation newly appeared in previously silent projection neurons and in presynaptic elements that had been silent. This presynaptic spike initiation was mediated by nitric oxide (NO) released from glial cells via the activation of glial mGluR1.

Materials and Methods

Dye injection. All animal studies were undertaken using protocols approved by the university animal ethics committee. Young (18–24 d of age) Sprague Dawley rats were placed in a stereotaxic apparatus under deep anesthesia with a mixture of ketamine and xylazine (8:1; 3.4 ml/kg). The skull was drilled to allow insertion of a 500 nl Hamilton syringe needle. A single injection of the voltage-sensitive absorption dye RH-482 (1 mg/ml) was administered into the right PAG. The injected rats survived for 5–7 d before slice preparation.

Preparation. For optical imaging of gross neural excitation, transverse slices (500 μ m thick) with dorsal roots attached (5–10 mm in length) were prepared from lumbosacral enlargements of 18- to 30-d-old Sprague Dawley rat spinal cords in cold Ringer solution. A slice was stained in a bath filled with RH-482 (0.1 mg/ml; 20 min) and set in a submersion-type chamber (0.2 ml) on an inverted microscope (IMT; Olympus, Tokyo, Japan) equipped with a 150 W halogen lamp. The slice was perfused with Ringer solution containing the following (in mM): 124 NaCl, 5 KCl, 1.2 KH_2PO_4 , 1.3 MgSO_4 , 2.4 CaCl_2 , 26 NaHCO_3 , 0.2 thio-urea, 0.2 ascorbic acid, and 10 glucose (oxygenated with 95% O_2 and 5% CO_2) at room temperature (20–24°C).

Optical images of excitation in projection neurons were taken from slices obtained from rats whose brain stems had been injected with the dye. For optical imaging of excitation in primary afferent fibers, an unstained slice was set in a chamber, a dorsal root was suctioned into a pipette filled with the voltage-sensitive dye (0.1 mg/ml), and recording was started after 2–3 hr.

Optical recording. The light absorption change in a 0.83 mm square area in the dorsal horn at a wavelength of 700 ± 32 nm was recorded by an imaging system (Deltalon 1700; Fuji, Tokyo, Japan) with 128 ± 128 pixel photo sensors at a frame rate of 0.6 msec. Thirty-two single pulses were given to the dorsal root at a constant interval of 12–15 sec. Starting at 10 msec before each stimulus, the image sensor took 128 consecutive frames of the light-absorption images at a sampling interval of 0.6 msec. A reference frame, which was taken immediately before each series of 128 frames, was subtracted from the subsequent 128 frames. Thirty-two series of these difference images were averaged and stored in the system memory. We determined the initial frame by averaging the first 15 frames of the difference image and then subtracting this average from each of the 128 frames of the image data on a pixel-by-pixel basis to eliminate the effects of noise contained in the reference frame. The ratio image was then calculated by dividing the image data by the reference frame. In most cases, the ratio image was filtered by a three-point moving average over time. The dorsal root was stimulated by a glass suction electrode. The test stimulation that activated A and C fibers was a current pulse of 2 mA with duration of 0.5 msec, and conditioning was a repetitive stimulation of 2 Hz for 2 min of the same current pulse.

Drugs. The RH-482 (NK-3630) dye was obtained from Nippon Kanko Shikiso (Okayama, Japan). The D-2-amino-5-phosphonovaleric acid, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine, monofluoroacetic acid, 7-nitroindazole, spermine NONOate, bicuculline methiodide, and strychnine hemisulfate were from Sigma (St. Louis, MO). CNQX, (S)-(+)- α -amino-4-carboxy-2-methylbenzeneacetic acid (LY367385), and (S)-3,5-dihydroxyphenylglycine (DHPG) were from Tocris Cookson (Bristol, UK).

Statistics. Results were expressed as means $\pm$ SE. The paired *t* test or nonparametric ANOVA (Tukey–Kramer test) was used to exhibit statistical differences. The symbols refer to statistical significances of $**p < 0.05$ and $*p < 0.1$ and to an insignificance of $\#p > 0.1$.

Results

Net excitation of presynaptic and postsynaptic elements

The first series of experiments was to locate the region where LTP is present. We visualized gross neural excitation elicited at both

presynaptic and postsynaptic elements in the dorsal horn region of transverse cord slices stained with a voltage-sensitive dye, RH-482 (Ikeda et al., 1998, 2000). Neuronal excitation elicited by a test stimulus (a single pulse of C fiber-activating strength to the dorsal root) was facilitated after a CS (a repetitive dorsal root stimulation of 240 pulses at 2 Hz for 2 min) (Fig. 1). This CS has been shown to induce LTP of C-afferent synapses on lamina I neurons projecting to the PAG (Sandkühler and Ikeda, 2003). In contrast, a higher-frequency conditioning (100 Hz for 1 sec) induces LTP in neurons projecting to the parabrachial area (Ikeda et al., 2003). These areas in the brainstem are two major supraspinal targets for lamina I projection neurons (Spike et al., 2003), and firing properties of these projection neurons are distinct from each other (Ruscheweyh et al., 2004). In the dorsoventral distributions (Fig. 1B) and spatial averages over the area (Fig. 1D,E), this facilitation was more prominent in lamina I than in lamina II (277 ± 30 vs $142 \pm 9\%^{**}$; $n = 4$). Therefore, we focused our analysis on LTP in lamina I. The facilitation in lamina I was attributable to not only an augmentation of pre-existing excitation but also newly elicited excitation in previously silent regions (Fig. 1B,C). Some pixels that displayed no sign of excitation began to generate responses to the test stimulation after CS (Fig. 1C,*). Here, $**$ and $*$ refer to significances of $p < 0.05$ and $p < 0.1$, respectively, and $\#$ to an insignificance of $p > 0.1$.

Because the stimulations were given to a dorsal root through the suction pipette, all types of fibers, including both A and C fibers, in the dorsal root were activated. As shown previously, the optical response elicited by the stimulation of C fiber-activating strength is much larger than the response to the stimulation-activating A fibers only, ~ 10 times or larger (Ikeda et al., 1998). The magnitude of A-fiber response was usually 0.01% or less of the background light intensity, close to the noise level of the system. Therefore, the optical response to the test stimulation can be considered primarily of C-fiber origin, although we cannot exclude their possible interaction. In addition, because the whole slice was stained with the dye, the optical response represents the net membrane potential change along the thickness of the slice. That is, it may reflect action potentials, postsynaptic potentials, and membrane potential change in any cells, and the magnitude of optical signals may not exactly coincide with membrane potential change in a specific group of cells. Therefore, the optical recording with whole-slice staining has been used to analyze spatiotemporal properties of neuronal activities (Tanifuji et al., 1994; Ikeda et al., 1998) and changes in net neuronal activities (Ikeda et al., 2000).

Presynaptic excitation

To identify whether the facilitation of neuronal excitation was attributable to a presynaptic or postsynaptic mechanism, we next investigated the effects of CS on the excitation of presynaptic elements. We stained the primary afferent fibers anterogradely with voltage-sensitive dye in the suction pipette for dorsal root stimulation. The test stimulation elicited action potential-like or compound action potential-like optical signals throughout the superficial dorsal horn (Fig. 2A). We have shown previously that, when single-pulse dorsal root stimulation is used, the postsynaptic component of the optical response is primarily mediated by glutamate receptors (Ikeda et al., 1998). The contribution of other substances, such as peptides and ATP, is masked by large optical signals reflecting the evoked action potentials, or is under the resolution of the method. A slow optical response evoked by a high-frequency repetitive stimulation is sensitive to a substance P antagonist (Murase et al., 1999). As shown in Figure 2A, the

Figure 1. Facilitation of gross neuronal excitation after CS to the dorsal root. *A*, Optical responses elicited by a single-pulse test stimulation to the dorsal root immediately before CS (left images) and at 240 min after (2 Hz for 2 min; current pulses of 2 mA with a constant duration of 0.5 msec; right images). Images were taken from the area indicated by a square in the schematic drawing of a transverse slice in *B*. The time after the test stimuli is indicated at the left of each image. *B*, Spatial distributions of the optical responses before CS (black) and after (red) along three dorsoventral lines, *a–c*, in the schematic drawing. Note larger facilitation in lamina I than in lamina II. *C*, Time courses of optical responses in the pixels before conditioning (black) and after (red) exhibiting augmentation of pre-existing response and generation of new response in previously silent pixels (e.g., *). The traces are the responses of 5×6 pixels (at a 3-pixel interval on the image sensor) within the red rectangle on the schematic drawing in *B*. *D, E*, Long-term facilitation in the mean magnitudes of the optical responses at lamina I (purple) and lamina II (orange). Each magnitude is shown as the percentage control of the spatiotemporal average during a period of 10 msec after the onset of response in the area indicated by rectangle filled with purple (lamina I) and orange (lamina II) in the schematic drawing of a transverse slice in *B*. The mean control response without CS at lamina I (purple) and lamina II (orange) in three slices was also illustrated with black lines to exhibit the stability of optical response over 4 hr.

glutamate receptor antagonists D-AP-5 (50 μM) and CNQX (10 μM) failed to alter the test stimulus-induced optical signals recorded with the anterograde staining ($117 \pm 5\%$; $n = 5$). Therefore, the possible leak of the dye to postsynaptic elements was minimal, or at least under the resolution of the method. It is reasonable to assume that the optical signal recorded with the anterograde staining represents the sum of excitation that occurred at stained presynaptic elements, such as afferent fibers and the terminals, along the slice depth.

As was apparent in the dorsoventral distributions (Fig. 2*C*) and spatial averages over the area (Fig. 2*E*), presynaptic excitation was significantly augmented after CS in lamina I, and in lamina II to a lesser extent ($181 \pm 5\%$ vs $118 \pm 4\%$; $n = 5$). The facilitation of presynaptic excitation in lamina I was not blocked by D-AP-5 and CNQX (Fig. 3*A*) ($183 \pm 10\%$; $n = 4$). However, the facilitation was not present in a Ca^{2+} -free solution (Fig. 3*B*) ($113 \pm 6\%$; $n = 4$). To exclude the involvement of postsynaptic excitatory and inhibitory neurons, D-AP-5 (50 μM) and CNQX (10 μM) were added to the solution throughout the following series of presynaptic excitation measurements. The augmentation of optically recorded presynaptic excitation may be attributable to an increase of spiking or depolarized elements

and/or an increase in the spike height in some presynaptic elements along slice depth and does not directly represent an enhanced release of neurotransmitter(s).

Because a diffusible messenger NO mediates the presynaptic form of LTP in some central synapses (Arancio et al., 1996; Hawkins et al., 1998; Volgushev et al., 2000; Maffei et al., 2003), we investigated whether NO contributes to the observed presynaptic facilitation. The neuronal NO synthase (nNOS) inhibitor, 7-nitroindazole (7NI; 100 μM), partially suppressed the facilitation induction (Fig. 3*C*) ($173 \pm 11\%$; $n = 5$). The inducible NO synthase (iNOS) inhibitor, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT; 100 μM), significantly inhibited the facilitation induction (Fig. 3*C*) ($150 \pm 11\%$; $n = 4$). In the spinal dorsal horn, iNOS has been observed in glial cells (Maihofner et al., 2000). Monofluoroacetic acid (MFA) has been shown to selectively inhibit glial metabolism both *in vivo* and *in vitro* (Muir et al., 1986; Swanson and Graham, 1994; Hulsman et al., 2000). Treatment with MFA (5 mM) significantly inhibited the facilitation induction (Fig. 3*D*) ($113 \pm 3\%$; $n = 4$), whereas it did not affect the magnitude of the test stimulus-induced response ($99 \pm 2\%$; $n = 4$).

Figure 2. Facilitation of presynaptic excitation after CS to the dorsal root and the presynaptic response newly generated after CS. The afferent fibers and terminals were stained anterogradely with a voltage-sensitive dye from the dorsal root. *A*, Optical response in the superficial dorsal horn elicited by single-pulse test stimulation (left images) and the effects of D-AP-5 and CNQX application (right image). Images were taken from the area indicated by the black rectangle on the schematic drawing of a transverse slice. The traces were the spatially averaged time course of optical responses in control (black line) and in the presence of D-AP-5 and CNQX (green line) over the purple rectangular area on the schematic drawing. *B*, Optical response in the superficial dorsal horn before CS (left images) and the facilitation after CS (right images). Images were taken from the area indicated by the black rectangle on the schematic drawing of a transverse slice in *A*. The time after the test stimuli is indicated at the left of each image. *C*, Spatial distribution of the optical responses before CS (black) and after (red) along three dorsoventral lines, *a–c*, on the schematic drawing. Facilitation in lamina I was more prominent than that in lamina II. *D*, Time courses of optical responses in the pixels before CS (black) and after CS (red). Responses were newly elicited in previously silent pixels after CS (e.g., *), and pre-existing responses were augmented. The traces show the responses of 4×3 pixels (at a 3-pixel interval on the image sensor) within the red rectangle in the schematic drawing in *A*. *E*, Facilitation of the mean magnitudes of the presynaptic optical responses after CS at lamina I (purple) and insignificant changes in those at lamina II (orange). Each magnitude is shown as the percentage control of the spatiotemporal average during a period of 5 msec after the onset of response. The respective averaged areas are indicated by the rectangle filled with purple and orange in the schematic drawing in *A*.

We further examined the mediator of NO liberation from glial cells. As shown in Figure 3*E*, the facilitation of presynaptic excitation after CS was significantly reduced by treatment with the mGluR1-selective antagonist LY367385 ($10 \mu\text{M}$; $112 \pm 3\%^*$; $n = 4$). In addition, the application of a group I mGluR-selective agonist, DHPG ($100 \mu\text{M}$), alone facilitated the presynaptic excitation ($168 \pm 6\%^*$; $n = 4$), and the facilitation induction was partially inhibited by MFA (Fig. 3*F*) ($118 \pm 3\%^*$; $n = 4$). Although a shorter period (30 sec) of CS or the application of the NO donor, spermine NONOate ($100 \mu\text{M}$), for 30 min alone did not facilitate the presynaptic excitation (Fig. 3*G*, 30 sec CS: $112 \pm 10\%^*$; $n = 4$) (Fig. 3*H*, NO donor: $105 \pm 8\%^*$; $n = 4$), a shorter (30 sec) CS in the application of spermine NONOate for 30 min facilitated the presynaptic excitation in MFA and LY367385 (Fig.

3*I*) ($160 \pm 13\%^*$; $n = 4$). These results suggest a possible contribution of mGluR1 on glial cells for the facilitation induction. Because some facilitation remained in the presence of AMT, MFA and LY367385 and also 7NI were effective (Fig. 3*C,D,E*), a possibility remains that LTP via other mechanism(s) coexisted in a lesser extent.

Interestingly, the conditioning not only increased the responses of each pixel but also elicited responding pixels that were silent before CS, particularly in lamina I and in a much lesser extent in other laminae, as was clearly exhibited by the dorsoventral distributions (Fig. 2*C*) and the optical signal of each pixel (Fig. 2*D*). This phenomenon indicates that new active primary afferent terminals may be generated after CS. The generation of new active terminals was clearly visualized by subtraction of re-

Figure 3. Facilitation of presynaptic excitation in lamina I after CS to the dorsal root under various conditions. Facilitation was not inhibited by excitatory amino acid receptor antagonists D-AP-5 and CNQX (*A*). The induction was inhibited in a Ca^{2+} -free solution (*B*) and by the application of nNOS inhibitor 7NI (*C*, filled circle), iNOS inhibitor AMT (*C*, open circle), glial metabolism inhibitor MFA (*D*), and mGluR1 antagonist LY367385 (*E*). A 15 min application of group I mGluRs agonist DHPG alone facilitated the presynaptic excitation (*F*, filled circle), and the facilitation was significantly less in the presence of glial metabolism inhibitor

Figure 4. Percentage changes in the number of responding pixels (mean $\pm$ SE) associated with the facilitation of presynaptic excitation under various conditions. The conditions are identical to those in Figure 3. The number of pixels over a certain threshold in control was subtracted from that after CS or drug application. An example of the counting procedure is illustrated in the insert for a conditioning stimulation of 2 min. Pixels showing an optical response over the threshold of 0.003% in control were selected (left), and the number was subtracted from that after CS (center). The difference image (right) exhibits the area where a response was newly generated by CS.

sponding pixels before and after CS, as shown in Figure 4 ($117 \pm 4\%^*$; $n = 4$). The new generation of responding pixels was not blocked by D-AP-5 and CNQX ($121 \pm 6\%^*$; $n = 4$) but was suppressed in a Ca^{2+} -free solution ($105 \pm 2\%^*$; $n = 4$). The increase of responding pixels was suppressed primarily by AMT ($98 \pm 6\%^*$; $n = 4$) and MFA ($102 \pm 6\%^*$; $n = 4$) but not by 7-NI ($114 \pm 6\%^*$; $n = 5$). The increase of responding pixels after conditioning was significantly reduced by LY367385 ($107 \pm 3\%^*$; $n = 4$). The application of DHPG alone increased the responding pixels ($117 \pm 7\%^*$; $n = 4$), and the increase of responding pixels was partially inhibited by MFA ($107 \pm 1\%^*$; $n = 4$). A shorter (30 sec) CS in the application of spermine NONOate for 30 min increased the responding pixels in the treatment with MFA and LY367385 ($113 \pm 6\%^*$; $n = 4$).

Postsynaptic excitation

If the LTP of afferent synapses is attributable to, in part, an enhanced presynaptic excitation, EPSPs recorded from postsynaptic cells should be potentiated with pharmacology similar to that of the presynaptic facilitation. Therefore, we recorded optically

MFA (*F*, open circle). A shorter CS (30 sec; *G*) or the application of NO donor alone (*H*) did not facilitate presynaptic excitation. A shorter CS in the presence of NO donor for 30 min facilitated presynaptic excitation. The degree of facilitation (mean $\pm$ SE) in these conditions at 75 min after stimulus is summarized in a bar graph (*J*).

the neuronal excitation of projection neurons that were retrogradely stained with a voltage-sensitive dye injected into the PAG. Because only several stained cells were found within lamina I in each slice, we were able to record the intracellular potential from an individual identified single neuron over several hours in a noninvasive manner. The test stimulation of single pulses to the dorsal root elicited either action potential-like optical signals (Fig. 5*B*) or EPSP-like optical signals (Fig. 5*C*) of projection neurons in lamina I. Both types of signals were mediated by the excitatory neurotransmitter, glutamate, because D-AP-5 (50 μ M) and CNQX (10 μ M) effectively eliminated the generation (Fig. 5*C*) ($11 \pm 3\%^*$ of control response; $n = 4$). Action potential-like optical signals, which were three to four times larger in magnitude than EPSP-like signals, were generated in approximately one-quarter of recorded cells as stimulus intensity increased. In the following experiments, we excluded the neurons that generated action potential-like optical signals.

The EPSP-like signals in projection neurons were augmented significantly after CS (Fig. 6*A*) ($226 \pm 11\%^*$; $n = 5$). The potentiation of EPSP-like signals was inhibited completely by AMT (100 μ M) and partially by 7NI (100 μ M) (Fig. 6*B*) (AMT, $94 \pm 6\%^*$, $n = 4$; 7NI, $167 \pm 9\%^*$). The effectiveness of a shorter (30 sec) CS period, which did not augment the EPSP-like signals (Fig. 6*A*) ($88 \pm 7\%^*$), was increased by the application of spermine NONOate (100 μ M) for 15 or 60 min (Fig. 6*D*) (15 min, $171 \pm 4\%^*$, $n = 4$; 60 min, $307 \pm 62\%^*$). Application of the NO donor without CS induced no change (Fig. 6*C*) ($91 \pm 7\%^*$; $n = 4$). Intriguingly, in four of the six slices that had shown no optical signals by the test stimulation, EPSP-like signals were generated after CS (Fig. 6*E*). These pharmacological properties were quite similar to those observed in presynaptic facilitation in the preceding section. However, note that this series of experiments does not exclude the presence of postsynaptic mechanisms for LTP in the projection neurons. In fact, the degree of postsynaptically recorded potentiation (226%) was greater than that of presynaptically recorded excitation (181%). Therefore, a postsynaptic form of LTP may also coexist with the presynaptic LTP.

Discussion

In this study, by using optical imaging, we reported that the CS facilitated gross excitation, presynaptic excitation, and postsynaptic excitation of identified projection neurons in the spinal dorsal horn lamina I. Because these facilitations were induced by the same CS and exhibited a similar NO dependency, facilitation of presynaptic excitation was the primary cause for the LTP observed in both presynaptic and postsynaptic recordings. Possible contribution of other presynaptic and/or postsynaptic form(s) of LTP was not excluded in this series of experiments.

LTP by glial nitric oxide via mGluR1 activation

The diffusible messenger NO is produced from L-arginine by three distinct isoforms of nitric oxide synthase: nNOS, iNOS, and endothelial NO synthase (eNOS). nNOS and eNOS are present in neurons, and they are involved in LTP induction of central synapses (Doyle et al., 1996; Kantor et al., 1996; Son et al., 1996; Hawkins et al., 1998). Recently, an LTP that is associated with an NO-dependent enhancement of presynaptic terminal excitability has been reported at the cerebellar mossy fiber–granule cell synapses by simultaneous recording of presynaptic and postsynaptic current changes (Maffei et al., 2003). In contrast, iNOS exists in glial cells, and its involvement in LTP function is poorly understood (Mori et al., 2001). In the spinal dorsal horn, although the presence of nNOS in neurons and of iNOS in glial cells has been

Figure 5. Optical response of postsynaptic PNs in lamina I. *A*, Optical images of a PN elicited by a single-pulse test stimulus to the dorsal root. The cell was retrogradely stained with a voltage-sensitive dye injected into the PAG of the brain stem. Images were taken from the area indicated by a rectangle on the schematic drawing of a transverse slice in *B*. The time after the test stimuli is indicated at the left of each image. *B*, An example of an action potential-like response of the same neuron shown in *A*. The trace is the spatially averaged time course of the optical response within the red area on the schematic drawing. *C*, EPSP-like optical response of a PN and the inhibition by D-AP-5 and CNQX.

reported previously (Maihofner et al., 2000), to our knowledge, their involvement to the induction of LTP has not been reported.

In this study, we demonstrated that an iNOS inhibitor, a glial metabolism inhibitor, and an mGluR1 antagonist inhibited LTP

Figure 6. Facilitation of the excitation in postsynaptic PNs after CS and the effects of NO-related agents. *A*, EPSP-like optical response in PNs was facilitated by CS for 2 min (filled circle) but not by CS for 30 sec (open circle). The top trace indicates an example of the optical response in a PN before and after CS (thin and bold traces, respectively). The bottom panels indicate the mean magnitudes of the optical responses in five PNs taken every 15 min. Each magnitude is shown as the percentage control of the spatiotemporal average during a period of 5 msec after the onset of the response. *B*, The facilitation by CS was inhibited by 7NI (filled circle) and AMT (open circle). *C*, The application of NO donor alone did not facilitate the optical response in PNs. *D*, Application of NO donor, NONOate, for 15 min (open circle) or 60 min (filled circle) with CS for 30 sec facilitated the optical response in PNs. The horizontal bars indicate the times when NONOate was applied. *E*, Optical response of PNs newly generated after CS. Time traces are the spatially averaged optical response of a PN before and after CS (thin and bold traces, respectively).

of presynaptic terminals, suggesting that NO may be produced in glial cells via the activation of mGluR1s. The result that LTP was induced in presynaptic terminals by a group I mGluR agonist further supports this mechanism. LTP of afferent-evoked EPSPs recorded in dorsal horn neurons has been reported to be inhibited by mGluR1 antagonists and is induced by the agonist (Zhong et al., 2000). Also, an increase of spontaneous and miniature EPSCs in spinal dorsal horn neurons by the group I mGluR agonist has been reported previously (Park et al., 2004). CS for 30 sec, which was ineffective alone, induced LTP by the application of an NO donor in the presence of antagonists for NMDA, non-NMDA glutamate, and mGluR1s and a glial metabolism inhibitor. Therefore, glial NO activated primary afferent fibers and/or the terminals directly, rather than acting on postsynaptic neurons

and/or glial cells. As mentioned in the Results, however, the nNOS inhibitor depressed the presynaptic and postsynaptic facilitation, and some facilitation remained in the presence of glial metabolism inhibitor, iNOS inhibitor, and mGluR1 antagonist. And also, the degree of postsynaptically recorded potentiation was greater than that of presynaptically recorded excitation. Therefore, other form(s) of LTP may also coexist or cooperate with the presynaptic LTP. Additional study is necessary to reveal the role of CS in the production of NO and the associated intracellular mechanisms.

LTP by presynaptic facilitation

An interesting finding in the gross excitation recording was that CS expanded the excited area in addition to the potentiation of pre-existing neuronal excitation. The expanded excited area might include CS sensitized neurons and/or primary afferents that had not been responding before CS. We showed that after CS, a new postsynaptic response was elicited in projection neurons that showed no response before CS. In addition, in the recording of excitation in presynaptic elements, responses were newly generated in previously silent areas after CS. This result suggests new spike generation in afferent terminals. It is reasonable to assume that such an occurrence of spikes in presynaptic terminals leads to the generation of active projection neurons that were silent before CS. The conversion of previously silent synapses into active ones has been reported to underlie LTP (Liao et al., 1995; Li and Zhuo, 1998; Malenka and Nicoll, 1999; Luscher and Frerking, 2001; Isaac, 2003; Kim et al., 2003; Malinow, 2003; Voronin and Cherubini, 2003). The rapid insertion of excitatory amino acid receptors in the postsynaptic membrane (Liao et al., 1995; Li and Zhuo, 1998; Malenka and Nicoll, 1999; Luscher and Frerking, 2001; Isaac, 2003; Malinow, 2003) or the initiation of presynaptic transmitter release (Kim et al., 2003; Voronin and Cherubini, 2003) is thought to mediate this response. The present study suggests another possible mechanism for the activation of silent postsynaptic neurons by the facilitation of presynaptic excitation.

In the spinal dorsal horn, the regulation of presynaptic spiking is known in the context of presynaptic inhibition. The silent presynaptic afferent terminals may become activated after their liberation from the shunting of action potentials at the terminals (Cattaert and El Manira, 1999; MacDermott et al., 1999; Parnas et al., 2000; Cattaert et al., 2001; Lee et al., 2002) or from the conduction block of action potentials by inhibitory transmitters at the branching points (Wall, 1995). This study has exhibited that such liberation from the shunting of presynaptic spiking might also underlie LTP.

Possible contribution to hyperalgesia

The involvement of both nNOS and iNOS in the dorsal horn to the hyperalgesia has been reported previously (Wu et al., 2001; Tao et al., 2003). In addition, mGluRs and glial cells have been shown to be involved in hyperalgesia *in vivo* (Meller et al., 1994; Watkins et al., 2001; Dolan and Nolan, 2002; Dolan et al., 2003). LTP, in this study, may underlie such NO-mediated and/or glial-related hyperalgesia. However, additional study is necessary to reveal the link between them.

The increase of spiking presynaptic elements after CS revealed in this study offers an explanation for the gradual increase in sensitivity and the expansion in receptive field observed in the secondary hyperalgesia (Meyer et al., 1994; Ziegler et al., 1999). In secondary hyperalgesia, excessive transmission of pain signals

from a wider area of skin is conveyed to the brain, resulting in abnormal pain sensitivity and memorizing the painful event. In fact, LTP induced by a low-frequency electrical stimulation to the skin (1 Hz) has been reported recently to spread to the adjacent skin region, leading to the induction of secondary hyperalgesia in human (Klein et al., 2004).

For further understanding, the visualization of single fiber excitation, simultaneous recording of excitation in fiber and single projection neurons, and examination of the involvement of NO in secondary hyperalgesia *in vivo* are necessary.

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