

Protein Synthesis Inhibition Blocks the Late-Phase LTP of C-Fiber Evoked Field Potentials in Rat Spinal Dorsal Horn

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Hu, Neng-Wei, Hong-Mei Zhang, Xiao-Dong Hu, Ming-Tao Li, Tong Zhang, Li-Jun Zhou, and Xian-Guo Liu. Protein synthesis inhibition blocks the late-phase LTP of C-fiber evoked field potentials in rat spinal dorsal horn. *J Neurophysiol* 89: 2354–2359, 2003; 10.1152/jn.01027.2002. Previous studies have demonstrated that in the hippocampus the maintenance of long-term potentiation (LTP) requires de novo protein synthesis. To investigate the role of protein synthesis in the maintenance of LTP of C-fiber evoked field potentials in spinal dorsal horn, which may be relevant to hyperalgesia, protein synthesis inhibitor (either cycloheximide or anisomycin) was applied locally to the recording segments of spinal cord in anesthetized rats, 30 min prior to tetanic stimulation to the sciatic nerve. We found that both cycloheximide and anisomycin selectively inhibited late-phase maintenance of the spinal LTP but affected neither LTP induction nor baseline responses of C-fiber evoked field potentials. In the presence of cycloheximide, LTP of C-fiber evoked field potentials was $281.5 \pm 16.5\%$ ($n = 6$) of baseline 1 h after tetanic stimulation and the potentiation significantly decreased to $235.5 \pm 18.5\%$ at 145 min after tetanic stimulation ($P < 0.05$). Afterward, LTP of C-fiber evoked field potentials decreased continuously and at 270 min after tetanic stimulation reached $130.8 \pm 18.0\%$, which was no longer different from baseline ($P > 0.05$). Spinal application of anisomycin at 30 min before tetanic stimulation yielded similar results ($n = 6$). These results suggest that protein synthesis may be crucial for the late-phase maintenance of LTP of C-fiber evoked field potentials in spinal dorsal horn.

INTRODUCTION

Long-term potentiation (LTP), which is first described in the hippocampus (Bliss and Lomo 1973), refers to a long-lasting enhancement in efficacy of synaptic transmission and is believed to be a synaptic model of learning and memory (Bliss and Collingridge 1993). Besides the hippocampus, LTP has been also found in several other parts of nervous system. Some of them may be relevant to pathological processes (McEachern and Shaw 1999).

In response to intensive noxious stimulation, neurons in spinal dorsal horn become hypersensitive to subsequent stimuli (Ma and Woolf 1995; Woolf 1983). The phenomenon that is termed central sensitization is considered as a central mechanism underlying hyperalgesia, an increased response to noxious stimuli (Woolf and Salter 2000). Just like hyperalgesia, LTP of C-fiber evoked field potentials in spinal dorsal horn can be induced by electrical stimulation of afferent C-fibers (Liu and Sandkühler 1995, 1997), natural noxious stimulation on

peripheral tissue, or acute nerve injury (Sandkühler and Liu 1998). Accordingly, the spinal LTP is believed to be an attractive cellular model of injury-induced hyperalgesia (Sandkühler 2000).

In the hippocampus two forms of LTP have been characterized. One is *N*-methyl-D-aspartate (NMDA) receptor-dependent (Harris et al. 1984; Hernandez et al. 1994) and another is NMDA receptor-independent (Harris and Cotman 1986). The maintenance of NMDA receptor-dependent LTP is divided into two phases, an early-phase (1–3 h) and a late-phase (>3 h). It has been well established that the late phase but not the early phase of NMDA receptor-dependent LTP is protein synthesis-dependent (Bliss and Collingridge 1993; Frey et al. 1988; Krug et al. 1984). In the case of NMDA-independent LTP, the role of protein synthesis appears complicated. It has been shown that protein synthesis inhibitors selectively inhibit the late-phase maintenance of LTP in mossy fiber-CA3 synapses in vitro (Huang et al. 1994) but block induction of LTP in the same synapses in vivo (Barea-Rodriguez et al. 2000). LTP of C-fiber evoked field potentials in spinal dorsal horn is also NMDA receptor-dependent (Liu and Sandkühler 1995). But the roles of protein synthesis in the induction and the maintenance of the spinal LTP have not been established to date.

In the present work two protein synthesis inhibitors, cycloheximide and anisomycin, were tested. We found that both of them selectively inhibited the late-phase of LTP of C-fiber evoked field potentials but did not affect its induction, when applied locally onto spinal dorsal horn at recording segments 30 min prior to LTP induction.

METHODS

Surgical preparation

Adult male Sprague–Dawley rats (250–300g) were anesthetized with urethane (1.5 g/kg ip). The trachea was cannulated and the animal breathed spontaneously. A catheter was inserted into one external jugular vein for intravenous infusion of Tyrode's solution at a rate of 0.8–1 ml/h. The caudal artery was cannulated to continuously monitor blood pressure, which was maintained at 80 to 120 mmHg. A laminectomy was performed to expose the lumbar enlargement of the spinal cord. The left sciatic nerve was dissected free for electrical stimulation with silver chloride hook-electrodes. The rats were placed

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in a stereotaxic frame. All exposed nerve tissues were covered with warm paraffin oil in a pool made of skin flaps, except for those spinal segments onto which the drug was applied. The body temperature of the rats was maintained at 37–38° with a feedback-controlled heating blanket. At the end of the experiments, animals were killed with an overdose of urethane. All experiments were approved by the local animal care committee.

Measurement of evoked potentials

The electrophysiological recording of C-fiber evoked field potentials has been described elsewhere (Liu and Sandkühler 1995, 1997). Briefly, following electrical stimulation of the sciatic nerve with a bipolar silver chloride hook-electrode, field potentials were recorded with a tungsten microelectrode (impedance 0.5–1 M Ω), which was driven by an electronically controlled microstepping motor (Narishige Scientific Instrument Laboratory) at a depth of 100–500 μ m from the surface of the spinal cord in lumbar enlargement (L4 and L5 segments). An A/D converter card (DT2821-F-16SE, Data Translation Inc.) was used to digitize and store data in a Pentium computer at a sampling rate of 10 kHz. Single square pulses (0.5 ms duration, delivered every 1 min) delivered to the sciatic nerve were used as test stimuli. The strength of stimulation was adjusted to 1.5–2 times the threshold for C-fiber response. A tetanic stimulation (100 Hz, 40 V, 0.5 ms, 100 pulses given in 4 trains of 1-s duration at 10-s intervals) was used to induce LTP of C-fiber evoked field potentials. The distance from stimulating site at the sciatic nerve to the recording site in the lumbar spinal cord was approximately 11 cm.

Experiments were performed in four groups of rats. The first one was the drug and tetanus-treated group, in which either cycloheximide ($n = 6$) or anisomycin ($n = 6$) was applied onto the surface of spinal cord at the recording segments, and 30 min later a tetanic stimulation was delivered. The second one was the drug control group, in which cycloheximide ($n = 5$) or anisomycin ($n = 5$) was given but no tetanic stimulation was delivered. The third was the vehicle and tetanus-treated group ($n = 5$). In this group saline was applied 30 min before tetanic stimulation. In the fourth group ($n = 6$) only saline was applied but no tetanic stimulation was delivered (saline control group).

Compounds and drug treatment

Cycloheximide (Sigma) was dissolved in 0.9% NaCl at concentration of 20 μ g/ μ l. Anisomycin (2.4 mg, Sigma) was first dissolved in 15 μ l of 1 N HCl solution and then treated with 1 N NaOH to a pH of 7.0. The solution was subsequently diluted with 0.9% NaCl to a concentration of 12 μ g/ μ l. To perform the controlled superfusion of spinal cord, a small well on the cord dorsum at the recording segments was formed with 1.5% agar dissolved with normal saline.

Data analysis

The area of C-fiber evoked field potentials was determined off-line by parameter extraction (see Fig. 1C), which was implemented by DataWave. In each experiment responses to five consecutive test stimuli were averaged. The mean area of C-fiber evoked field potentials before drug or saline application served as baseline. All data are expressed as means \pm SE. For statistical analysis, data within animals were compared with the nonparametric Friedman test and Wilcoxon signed-rank test and data between animals were compared with Kruskal–Wallis test and Mann–Whitney U test, when appropriate. Nonparametric tests were performed because the data from some groups were not normally distributed. $P < 0.05$ was considered significant.

RESULTS

Saline affected neither spinal LTP nor baseline of synaptic transmission

Our previous work (Liu and Sandkühler 1997) has shown that tetanic stimulation of the sciatic nerve induces LTP of C-fiber evoked field potentials in spinal dorsal horn, which lasts until the end of the experiment (≤ 10 h after tetanic stimulation), when the recording segments of the spinal cord are covered with warm paraffin oil. Because the protein synthesis inhibitors used in the present study were dissolved in saline, we first tested whether saline by itself could affect LTP of C-fiber evoked field potentials or baseline synaptic transmission over a long period of recording time. In five rats the stable baseline of C-fiber responses was recorded for ≥ 30 min and then saline (150 μ l in volume) was applied directly onto the dorsal surface of the recording segments. Thirty minutes later a tetanic stimulation (40 V, 0.5 ms, 100 Hz) was delivered to the sciatic nerve. As observed in previous work, the tetanic stimulation induced a robust LTP of C-fiber evoked field potentials in every experiment. The potentiation was $268.8 \pm 20\%$ ($P < 0.05$, Wilcoxon signed ranks test, Fig. 1A) as measured 1 h after tetanic stimulation and lasted until end of each experiment (for ≥ 6 h after tetanic stimulation). In five other rats we observed that spinal application of saline (without tetanic stimulation) had no effect on the basal synaptic transmission throughout the time of the experiment ($P > 0.05$, Friedman test, Fig. 1B).

Both cycloheximide and anisomycin selectively inhibit the late-phase of spinal LTP with no effect on LTP induction and baseline of synaptic transmission

In six rats cycloheximide (20 μ g/ μ l, 150 μ l in volume) was applied directly onto the recording segments following ≥ 30 min stable recordings of C-fiber evoked field potentials. The application did not affect LTP induction by a tetanic stimulation (100 Hz, 40 V, 0.5 ms) delivered 30 min after the drug application. One hour after tetanic stimulation, the mean potentiation in the six rats was $281.5 \pm 16.5\%$ ($P < 0.05$, Wilcoxon signed-rank test, Fig. 2A), which was not different compared with that recorded in saline group ($268.8 \pm 20\%$, $P > 0.05$, Mann–Whitney U test), indicating that the drug does not affect the spinal LTP induction and its early phase maintenance. However, the spinal LTP in this group decreased with time. At 145 min after tetanic stimulation, LTP decreased to $235.0 \pm 18.5\%$, which was significantly lower compared with that at 60 min after tetanic stimulation in the same group of rats ($P < 0.05$, Wilcoxon signed-rank test) and was also significantly lower compared with that in the saline and tetanus-treated group ($293.9 \pm 15.5\%$, $P < 0.05$, Mann–Whitney U test, Fig. 3A), but still significantly higher compared with baseline ($P < 0.05$, Wilcoxon signed ranks test) and that in the drug control group described below ($P < 0.05$, Mann–Whitney test, Figs. 2 and 3A). This suggested that at this time point the spinal LTP was substantially depressed but did not reach baseline level yet. At 270 min after tetanic stimulation, LTP decreased to $130.8 \pm 18.0\%$, which was no longer different from baseline ($P > 0.05$, Wilcoxon signed-rank test) and was also not different compared with that recorded in the drug control group ($104.8 \pm 4.8\%$, $P < 0.05$, Mann–Whitney U test,

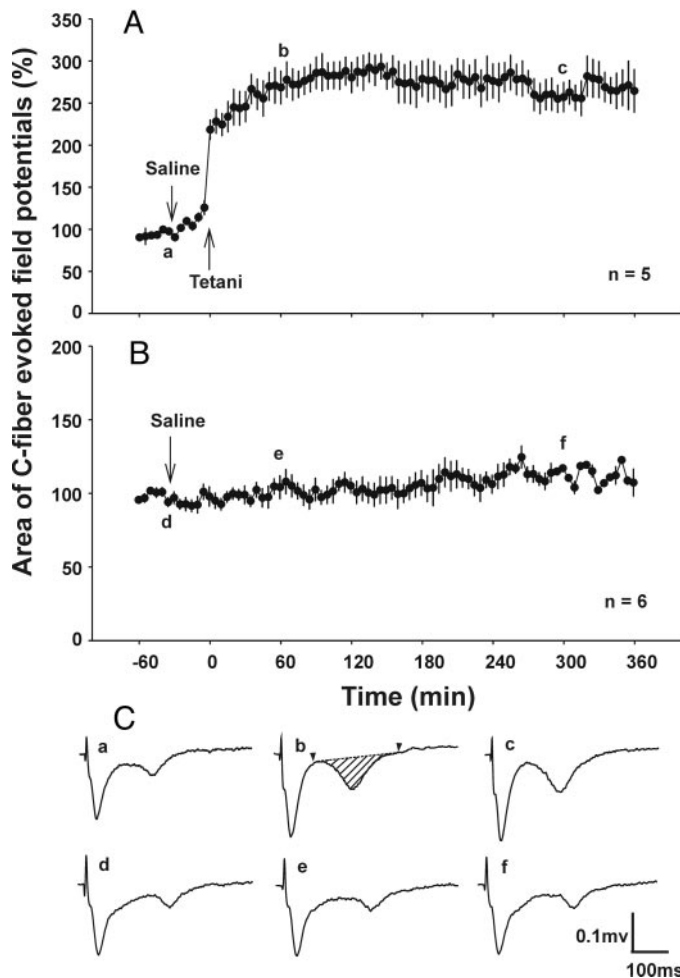


FIG. 1. Spinal application of saline affects neither spinal long-term potentiation (LTP) (A) nor baseline response of synaptic transmission (B). In each experiment the C-fiber responses recorded before saline application were averaged and served as baseline. Mean area of C-fiber evoked field potentials, expressed as a percentage of baseline, was plotted versus time. Vertical bars indicate SEs. Downward arrows indicate the onset of saline application on the surface of spinal dorsal horn at recording segments and upward arrow represents the time of tetanic stimulation (100 Hz, 40 V, 0.5 ms, 100 pulses given in 4 trains of 1-s duration at 10-s intervals). C: representative original recordings taken at the time points as indicated. Area of C-fiber evoked field potential (b; filled with oblique lines) is determined automatically by parameter extraction. Baseline (dashed line) is determined by two highest points within the time range that is defined manually on either side of C-fiber response (arrowheads). Therefore a small change in latency of C-fiber evoked field potentials does not affect the measurement of the area.

Figs. 2 and 3A), indicating the spinal LTP was not totally reversed until this time point.

In five other rats the same dosage ($20 \mu\text{g}/\mu\text{l}$, $150 \mu\text{l}$ in volume) of cycloheximide was administered to the spinal cord following ≥ 30 min stable baseline recordings. Recordings continued for ≥ 7 h without tetanic stimulation. The drug had no significant effect on the spinal basal synaptic transmission compared with the saline control group ($P > 0.05$, Kruskal-Wallis test, Figs. 1B and 2B).

To exclude the possibility that cycloheximide may inhibit spinal LTP through nonspecific effects other than inhibition of protein synthesis, we next examined the effect of anisomycin, another translation inhibitor on spinal LTP in six other rats. When anisomycin ($12 \mu\text{g}/\mu\text{l}$, $150 \mu\text{l}$ in volume) was applied to

the spinal cord 30 min before tetanic stimulation, a significant inhibition of spinal LTP also occurred, which was similar to that found with cycloheximide (Fig. 4A). At 60 min after tetanic stimulation, potentiation was $261.9 \pm 10\%$ ($P < 0.05$, Wilcoxon signed-rank test), which was not different from that in the saline and tetanus-treated group ($P > 0.05$, Mann-Whitney U test). At 125 min after tetanic stimulation, the spinal LTP decreased to $241.5 \pm 9\%$, which was significantly lower compared with that recorded at 1 h after tetanic stimulation in the same group of rats ($P < 0.05$, Wilcoxon signed-rank test) and was also significantly lower than that in the saline and tetanus-treated group ($287 \pm 17.6\%$, $P > 0.05$, Mann-Whitney U test, Fig. 3B). But the value was still higher than baseline ($P < 0.05$, Wilcoxon signed-rank test) and also higher than that recorded in the drug control group ($118.7 \pm 8.5\%$, $P < 0.05$, Mann-Whitney test, Fig. 3B). Until 295 min after tetanic stimulation the potentiation was totally reversed, since the mean area of C-fiber evoked field potentials de-

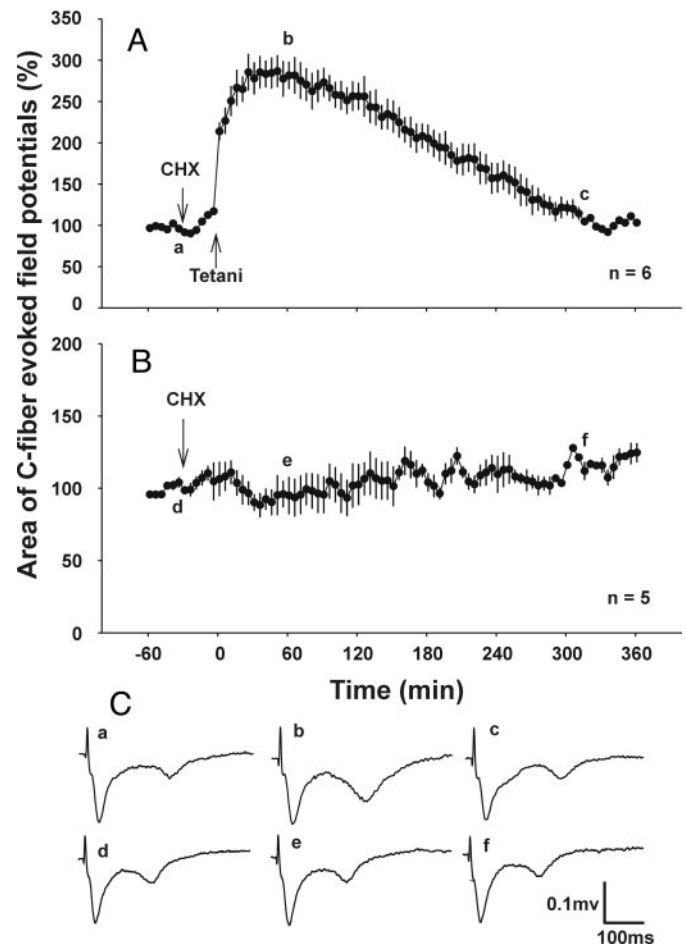


FIG. 2. Cycloheximide selectively inhibits the late phase of LTP of C-fiber evoked field potentials but affects neither the induction of the spinal LTP nor baseline response of C-fiber evoked field potentials. In each experiment the mean response recorded before drug application served as baseline. Mean area of C-fiber evoked field potentials, expressed as a percentage of baseline, was plotted versus time. Vertical bars indicate SEs. Downward arrows indicate the onset of drug application. Upward arrow represents the time of tetanic stimulation (100 Hz, 30–40 V, 0.5 ms). A: cycloheximide (CHX, $20 \mu\text{g}/\mu\text{l}$) does not influence the LTP induction but inhibits the late-phase maintenance of the spinal LTP. B: cycloheximide ($20 \mu\text{g}/\mu\text{l}$) has no effect on the baseline response of C-fiber evoked field potentials throughout the experiment period. C: representative original recordings taken at the time points as indicated.

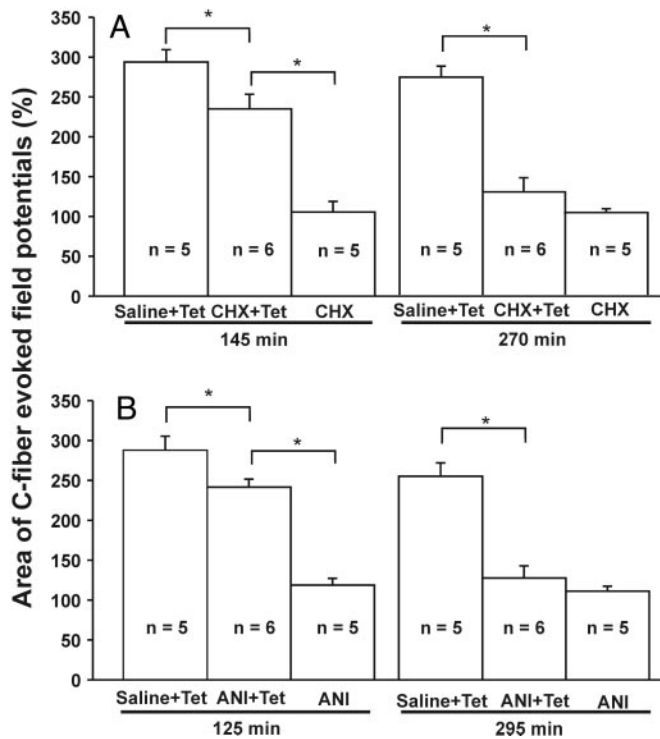


FIG. 3. Effects of saline, cycloheximide, or anisomycin on the maintenance of LTP of C-fiber evoked field potentials and basal synaptic transmission. *A*: at 145 min after tetanic stimulation, the spinal LTP in the cycloheximide and tetanus-treated group (CHX + Tet) significantly decreased compared with that recorded in the saline and tetanus-treated group (Saline + Tet), indicating a substantial depression of the spinal LTP. But it was still higher than that recorded in cycloheximide control group (CHX). At 270 min after tetanic stimulation, the mean area of C-fiber evoked field potentials was no longer different from that in the cycloheximide control group, indicating a total reversal of the spinal LTP. *B*: a significant depression of the spinal LTP in anisomycin and tetanus-treated group (ANI + Tet) was observed at 125 min after tetanic stimulation and at 295 min after tetanic stimulation the mean area of C-fiber evoked field potentials reached baseline. Vertical bars indicate SEs. * $P < 0.05$.

creased to $127.7 \pm 15.3\%$, which was not different from baseline ($P > 0.05$, Wilcoxon signed-rank test) and from that recorded in the drug control group ($111.1 \pm 6.4\%$, $P > 0.05$, Mann-Whitney U test, Fig. 3*B*).

The same dosage ($12 \mu\text{g}/\mu\text{l}$, $150 \mu\text{l}$ in volume) of anisomycin applied to recording segments had no significant effect on C-fiber evoked field potentials compared with the effect in the saline control animals. ($P > 0.05$, Kruskal-Wallis test, Figs. 1*B* and 4*B*). Thus the spinal application of anisomycin did not affect the baseline of synaptic transmission.

DISCUSSION

We found that inhibition of de novo protein synthesis by spinal application of cycloheximide or anisomycin before tetanic stimulation selectively inhibited the late-phase maintenance of LTP of C-fiber evoked field potentials but did not affect the induction and early-phase maintenance of the spinal LTP.

The results in the saline-treated group showed that LTP of C-fiber evoked field potentials induced by tetanic stimulation was stable until end of the experiments (≤ 6 h, Fig. 1*A*). When no tetanic stimulation was delivered, the baseline responses of

C-fiber evoked field potentials did not change during the whole recording period (Fig. 1*B*). These indicate that our experiment model is suitable to investigate the late phase of the spinal LTP.

The unspecific effects of the drugs may be not responsible for the present results, because both cycloheximide and anisomycin blocked late phase of the spinal LTP in the same manner, even with a similar time course. It is well known that the two drugs have a common effect, protein synthesis inhibition, but their side effects may be different. It has been reported that anisomycin can also strongly activate the mitogen-activated protein kinase subtypes (Cano et al. 1994; Hazzalin et al. 1998), while the significant side effect of cycloheximide has not been documented. We believe that the inhibitory effect observed in the present study is attributable to inhibition of protein synthesis in spinal recording segments.

It is unlikely that the drugs at the dosages used in the present work may depress the spinal LTP maintenance by toxic effects during the long experimental time course, because we showed

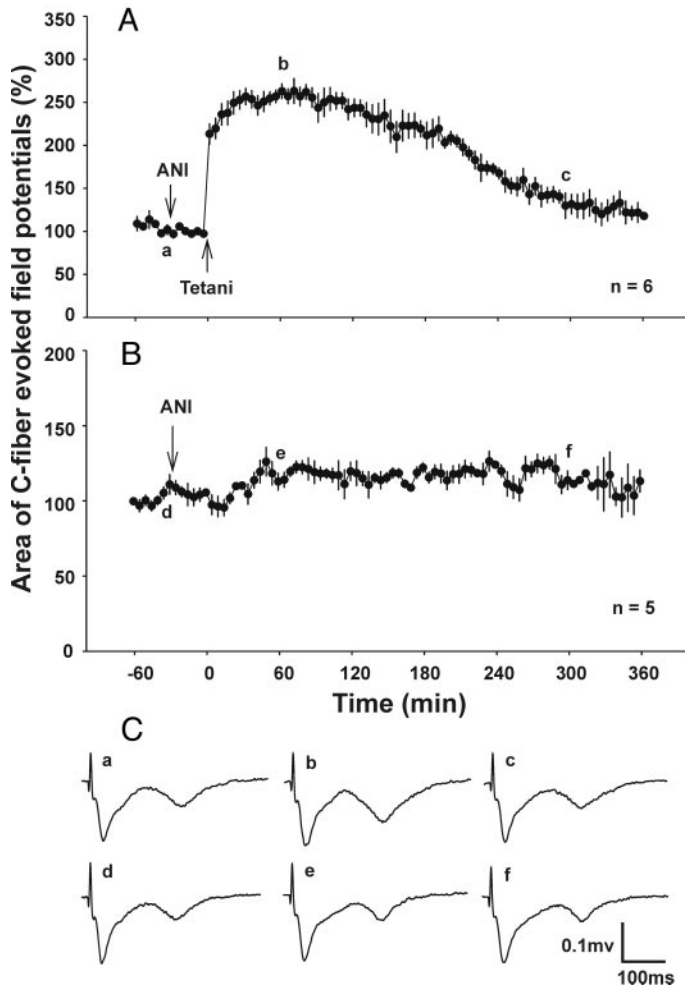


FIG. 4. Anisomycin selectively inhibits the late-phase of LTP of C-fiber evoked field potentials but affects neither the induction of the spinal LTP nor baseline response of C-fiber evoked field potentials. *A*: anisomycin (ANI, $12 \mu\text{g}/\mu\text{l}$) was applied on the surface of the spinal cord (downward arrow) and tetanic stimulation (100 Hz, 30–40 V, 0.5 ms) to the sciatic nerve was delivered 30 min later. *B*: spinal application of anisomycin ($12 \mu\text{g}/\mu\text{l}$) does not affect baseline response of C-fiber evoked field potentials. *C*: representative original recordings taken at the time points as indicated.

that cycloheximide or anisomycin at the concentrations capable of inhibiting LTP maintenance did not affect basal synaptic transmission elicited by test stimuli (Figs. 2B and 4B).

In the hippocampus, the late phase (>3 h) but not early phase (1–3 h) of NMDA receptor-dependent LTP depends on new protein synthesis (Frey et al. 1988; Krug et al. 1984; Mochida et al. 2001). The present work demonstrated that the situation was very similar in spinal dorsal horn. In the presence of cycloheximide or anisomycin, a significant depression of LTP of C-fiber evoked field potentials could be detected around 2 h after tetanic stimulation, and a complete reversal of the spinal LTP was observed around 4 h after tetanic stimulation (Fig. 3). Unlike the LTP mentioned above, the induction of some forms of long-lasting enhancement of synaptic transmission is protein synthesis-dependent, for instance, LTP induced by brain-derived neurotrophic factor and neurotrophin-3 in hippocampus slices (Kang and Schuman 1996), the LTP in hippocampal mossy fiber–CA3 synapses induced by tetanic stimulation (Barea-Rodriguez et al. 2000), the long-term facilitation (LTF) in spinal respiratory motor output induced by intermittent hypoxia (Baker-Herman and Mitchell 2002), and the LTF of synaptic transmission in cultured *Aplysia* sensory to motor neurons produced by repetitive application of serotonin (Martin et al. 1997). It has been proposed that LTP initiates the creation of a short-lasting (<3 h) protein synthesis-independent synaptic tag, and the tagged synapses can capture the proteins that are synthesized in soma and exported throughout the cell. In this way, newly synthesized proteins, which are crucial for late-phase LTP, are transported and specifically targeted to the synapses that are stimulated during LTP induction (Frey and Morris 1997). This theory may explain the fact that the late-phase but not the early-phase of LTP is inhibited by protein synthesis inhibitors (Frey and Morris 1998). Another source of new proteins is locally at the synaptic sites. It has been demonstrated that synapse-associated polyribosome complexes are selectively localized beneath postsynaptic sites on the dendrites of CNS (Steward and Levy 1982). The translation machinery may synthesize key molecular constituents of the synapse in response to activation of synapse (Steward et al. 2001). The proteins synthesized in dendrites are believed to contribute to the induction or early-phase maintenance of some forms of LTP (Barea-Rodriguez et al. 2000; Kang and Schuman 1996). It is likely that proteins synthesized in the cell body and in dendrites may play different roles in LTP maintenance. It has been shown that the α -subunit of calcium/calmodulin-dependent protein kinase II (CaMKII) is synthesized in the dendrites (Scheetz et al. 2000) and activation of CaMKII is proved to be necessary for induction but not for maintenance of LTP in the hippocampus (Lisman et al. 2002). Our unpublished data showed that CaMKII inhibitor (KN-93) blocked induction of LTP of C-fiber evoked field potentials when applied before LTP induction and reversed the established spinal LTP in a time-dependent manner. Within 1 h after LTP induction, KN-93 reversed LTP completely, but did not affect LTP when applied 3 h after LTP induction, suggesting that early phase but not late phase of the spinal LTP may depend on the activation of CaMKII. The new proteins involved in the late-phase maintenance of the spinal LTP remain to be elucidated.

It is generally accepted that LTP in the hippocampus, a brain structure associated with memory (Scoville and Milner 1957), is a synaptic model of learning and memory (Bliss and Col-

lingridge 1993). In recent years different forms of LTP in spinal dorsal horn have been demonstrated both in vivo (Liu and Sandkühler 1995; Svendsen et al. 1999) and in vitro (Randic et al. 1993). It is well established that nociceptive A δ - and C-fibers make the first synaptic contact with neurons in the spinal dorsal horn (Gobel and Falls 1979; Gobel et al. 1981; Light and Perl 1979). Therefore the spinal LTP mediated by afferent A δ - or C-fibers is suggested as a synaptic model for pain memory, which may be relevant to central sensitization, a central component of hyperalgesia (Liu and Sandkühler 1997; Melzack et al. 2001; Sandkühler 2000; Zimmermann 2001). This hypothesis is strongly supported by the fact that acute injury of the sural nerve or intensive noxious stimulation of peripheral tissues induces the spinal LTP in spinalized rats (Sandkühler and Liu 1998). Our recent work (in preparation) further demonstrates that acute injury of the sciatic nerve produces the spinal LTP in intact rats. Furthermore, we have shown that LTP-inducing tetanic stimulation delivered to the sciatic nerve produces mechanical and thermal hyperalgesia that lasts for several days (Zhang et al. 2002). The new proteins synthesized during LTP induction may play a role in the long-lasting abnormal pain behaviors.

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