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NALOXONE BLOCKS LONG-TERM DEPRESSION OF EXCITATORY TRANSMISSION IN RAT CA1 HIPPOCAMPUS *IN VITRO*

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INTRODUCTION

Several forms of LTD have been observed in the hippocampus, including heterosynaptic (24, 41), homosynaptic (14, 30) and associative LTD (6, 21, 37). Despite numerous studies on LTD, the mechanism(s) of induction and maintenance are still unclear.

Synaptic depression can be induced by strong tetanic stimulation with intensities higher than test stimuli, especially applied to the SCC fiber projections to CA1 pyramidal neurons. Bashir and Collingridge (2) reported that the specific NMDA receptor antagonist AP5 prevented the induction of homo- and heterosynaptic depression. In contrast, Paulsen *et al.* (33) showed that the transitory depression that followed strong tetanic stimulation of SCC fibers persisted in the presence of AP5 during the tetanic stimulation.

However, the strong stimulation parameters for LTD induction may activate local circuits and/or afferent systems other than SCC fibers. One such possible target is the PP fiber system from the entorinal cortex (EC). Anatomical studies have demonstrated that small axons originating from pyramidal cells located in layer III of the EC travel via PP and terminate in stratum lacunosum moleculare of the CA1 region (16, 42). In an ultrastructural study, Desmond *et al.* (13) showed that EC-CA1 axons form synapses primarily with dendritic spines of pyramidal cells and only rarely on dendrites of non-pyramidal or inhibitory neurons. More recently, electrophysiological studies *in vitro* (15) and *in vivo* (20) showed that stimulation of the PP evoked fEPSPs in stratum lacunosum moleculare of CA1.

It is known that glutamate exists in PP axons. However, enkephalin-like immunoreactivity has been co-localized within PP terminations in the stratum lacunosum moleculare and in the vicinity of the SCC-CA1 synapses (5, 16, 27). In addition, several reports (7, 27) have shown that multiple opioid receptor subtypes are localized in the CA1 region. Therefore, we hypothesized that strong tetanic stimulation of SCC to induce LTD might release opioids from PP and/or local interneurons, and that these opioids may provide the cellular mechanisms underlying LTD. Here we report that high-intensity generated LTD of CA1 fEPSPs is blocked by the opiate antagonist, naloxone, but not by the NMDA receptor antagonist AP5.

METHODS

Preparation.

We anesthetized male Sprague Dawley rats (120 to 200 g) with halothane (3%), and rapidly removed their hippocampal formations. We then cut transverse hippocampal slices of 400 μm thickness on a vibroslicer (Campden Instruments) and placed them in ice-cold (6 to 10° C) artificial cerebrospinal fluid (ACSF), gassed with 95% O_2 / 5% CO_2, of the following composition (in mM): NaCl (130); KCl (3.5); NaH_2PO_4 (1.25); $MgSO_4 \cdot 7H_2O$ (1.5); $CaCl_2$-$2H_2O$ (2); NaHCO3 (24); Glucose (10). After 60 min of pre-incubation at room temperature, the slices were transferred to a recording chamber in an 'interface' configuration and continuously perfused with ACSF at a constant rate (2 - 4 ml/min) for the remainder of the experiment. The recording chamber had a total volume of about 0.5 ml; at the superfusion rates used, 90% replacement of the chamber solution could be obtained within 1 to 2 min (37). During testing we maintained the bath temperature constant at 34°C.

Electrophysiology.

Methods of superfusion, drug administration and data analysis were as described previously (3). We used glass micropipettes filled with NaCl (3M; tip resistances 4 - 6 MΩ) led to an Axoclamp 2A (Axon Instruments) to record fEPSPs of CA1 neurons. Voltage records were filtered at 10 KHz, acquired by D/A sampling and acquisition software (pClamp; Axon Instruments), and stored on a personal computer system. We quantified (off-line data analysis via Clampfit; Axon Instruments) the field responses by measuring the initial slope of the fEPSPs using linear regression over a brief interval (300 μs) 4 ms after the stimulus artifact. Slope values were normalized across experiments by determining the mean of the preconditioning baseline response as 100% of control. The normalized slopes were averaged within control and experimental groups. The initial slope of the fEPSP represents an accurate estimation of the excitatory synaptic strength, considering its linear relationship with the synaptic conductance (19) and the minimization of the contribution of feedback inhibitory postsynaptic potentials (IPSPs) and changes in spike properties.

We elicited SCC-evoked fEPSPs with a bipolar stimulating electrode placed into the stratum radiatum, at least two-thirds of the distance from stratum pyramidale to stratum lacunosum moleculare (see Fig. 1A). The test stimulation consisted of a brief 50 μs constant current (150 - 200 μA) square pulse. At the start of each experiment we selected the stimulation intensity for baseline measurement to yield between one half to two-thirds of the maximal response. The fEPSP measures were an average of 5 sweeps collected every 5 min. The slice was stimulated every 20 s for the duration of each experiment to prevent variability in the fEPSP response, We induced LTD (defined as < 80% of baseline fEPSP at 20 min post-tetanus) of CA1 fEPSPs by strong (intensity: 3 - 5 times the test stimulus) tetanic stimulation consisting of 2 trains of 100 Hz for 1 s, at an interval of 20 s, delivered to the SCC fibers (LTD stimulation). Long-term potentiation (LTP; > 120% of control at 20 min post-tetanus) was induced with tetanic stimuli consisting of 2 trains of 100 Hz for 1 s with an interval of 20 s at the test stimulus intensity (LTP stimulation).

Drug treatment.

We added drugs from concentrated stock solutions to the ACSF in known concentrations immediately before administration to the slice chamber. The usual testing protocol was: recording of fEPSPs for 20-25 min during superfusion of ACSF alone (control), or with drugs (AP5 or naloxone) followed by tetanic (LTD or LTP) stimulation with additional recording of the fEPSPs for 15 min or longer. We obtained AP5 from Research Biochemical and naloxone from Sigma.

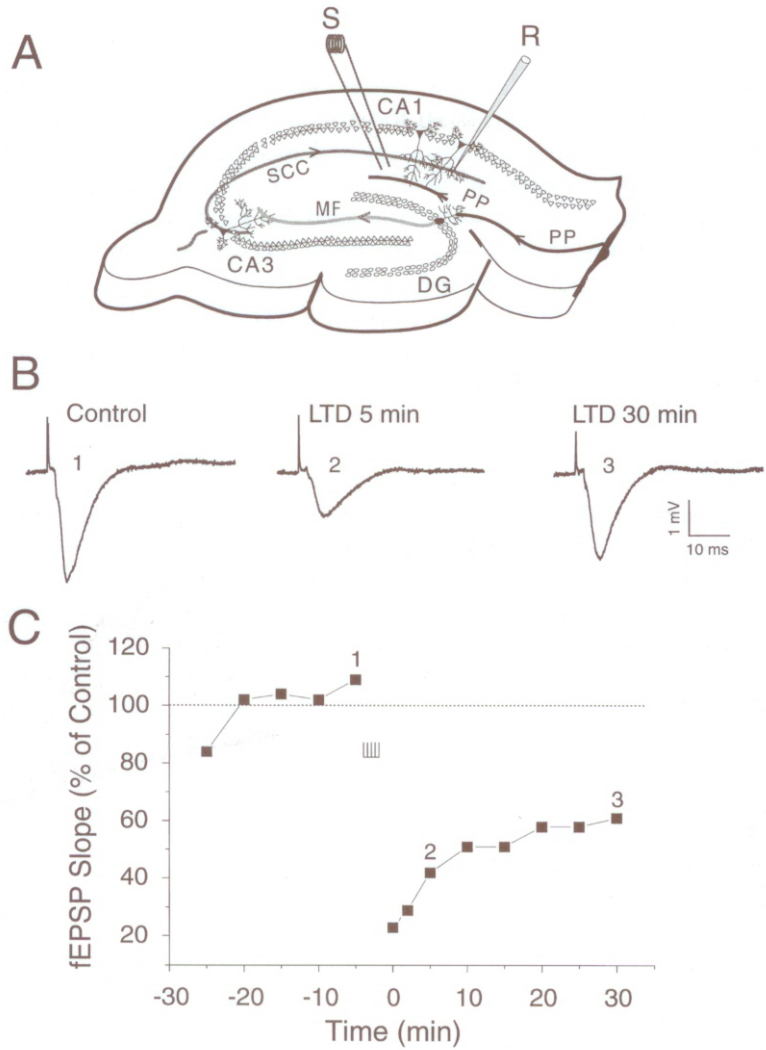

Fig. 1. - *Strong stimulation to SCC elicits LTD of fEPSPs in the CA1 region.*

A: Drawing of rat transverse hippocampal slice showing placements of recording (R) and bipolar stimulating electrodes (S). SCC = Schaffer collateral-commissural pathway; PP = Perforant path; MF = mossy fiber; and DG = dentate gyrus. B: fEPSPs evoked by SCC stimulation. In control conditions a single stimulus (50 μs, 150 μA) applied to the SCC region elicited a negative-going fEPSP. Strong tetanus (2 trains at 100 Hz, 20 s apart at 3 times the test stimulus = LTD stimulation) to SCC region decreased fEPSP slope and amplitude at 5 and 30 min. The number over each voltage trace represents the response for the graph in C. C: Graph of one experiment (same slice as in B) expressed as fEPSP slope, % of control. The dashed line indicates 100% of control; symbol = delivery of tetanus trains.

Data analysis.

Data are expressed as mean ± standard error of the mean (S.E.M.). For statistical analysis we used one way ANOVA with repeated measures. We considered $p < 0.05$ as significant.

RESULTS

We recorded from 53 hippocampal slices taken from 20 rats. Figure 1B shows negative-going fEPSPs recorded from the apical dendritic region of CA1 neurons in response to a single-pulse stimulus applied to SCC fibers. The fEPSP latency was 7 - 7.5 ms from stimulation onset to maximum amplitude. In most fEPSP recordings, presynaptic volleys were too small to be measured (Fig. 1B). In the slice of figure 1B, strong tetanic stimulation (LTD stimulation) induced synaptic depression of the fEPSP persisting longer than 30 min (Fig. 1C). Such LTD tetanic stimulation applied to the SCC fibers induced LTD of fEPSPs (i.e., initial slope < 80% of control) in 17 of 20 slices; at 2 min post-tetanus the fEPSPs decreased to 26 ±7%, at 20 min to 57 ± 8%, and at 40 min to 72 ± 10% of control (Fig. 2). In 5 slices, we recorded the fEPSPs for longer times and observed a full recovery of the fEPSP depression at around 60 min. In 3 slices, LTD stimulation only depressed the fEPSPs for 5 -10 min. In contrast, tetanic stimulation of the SCC fibers at the test intensity (LTP stimulation) resulted in LTP of the slope fEPSPs (initial slope > 120 % of control) in 6 of 6 slices (Fig. 2). Thus, in the same pathway, LTP or LTD can be generated by using the same stimulation frequency and duration but different intensities; at test intensity the tetanus gives rise to LTP, while at 3 - 5 times the test intensity it results in LTD.

It has been reported that transient shifts in DC potential (15 - 20 mV) of variable duration (40 - 60 s) were associated with LTD evoked by tetanic stimulation of

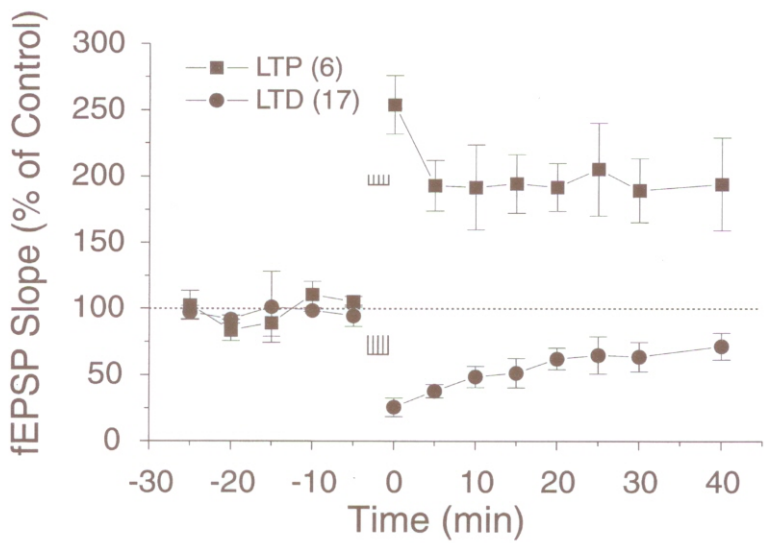

Fig. 2. - *LTP and LTD of fEPSPs, shown as averaged responses induced by different tetanic stimulations applied to the SCC region.*

In 6 slices, low intensity LTP stimulation to the SCC region elicited LTP of fEPSPs. In contrast, in 17 different slices, stronger tetanus (LTD stimulation; see Methods) to the SCC region induced LTD of fEPSPs. Dashed line indicates 100 % of control; symbol =tetanus trains; error bars = S.E.M.

SCC (2). In the present study, in 12 slices we also observed a similar DC potential shift associated with LTD stimulation-induced depression of fEPSPs. However, in 5 other slices where clear LTD of fEPSPs was established, we observed no DC potential shift. It has been reported that some form of LTD is mediated by NMDA receptors (14, 30). Therefore, we tested NMDA receptor involvement in the present study. In 6 slices, superfusion before tetanus of 50 μM AP5, the NMDA receptor antagonist, did not block the DC shift, and in all 6 slices LTD stimulation still induced LTD of fEPSPs, at 20 min (45 ± 8% of control) and at 40 min (80 ± 10% of control) post-tetanus (data not shown).

We investigated the possible role of opioid peptides on LTD of fEPSPs in 16 slices. Figure 3A shows fEPSP LTD from one slice superfused with normal ACSF (left panel), after a strong tetanus applied to the SCC pathway. At 20 min post-tetanus the fEPSP was still depressed to 68% of control. In the same slice, at 20 min post-tetanus the stimulus strength was increased to bring the fEPSP slope value back to control level. Superfusion of 1 μM naloxone for 30 min alone had no effect on the initial slope of the fEPSP. However, naloxone superfusion prevented the subsequent induction of a second LTD (at 20 min post-tetanus; 97% of control; Fig. 3A; right panel). The average of 5 such experiments are shown in figure 3B. In control solution, the first LTD stimulation of SCC fibers induced a depression of the initial slope of fEPSP at 2 min (23 ± 12% of control), at 5 min (34 ± 7% of control), at 10 min (49 ± 8% of control), and at 15 min (57 ± 8% of control) post-tetanus. After 1 μM naloxone perfusion (30 min), we applied a second LTD stimulation and only a short-lived depression of fEPSPs occurred, at 2 min post-tetanus (43 ± 12% of control); at 5 min it was 81 ± 7% of control, at 10 min it was 81 ± 8%, and at 15 min it was 93 ± 5%. Naloxone significantly blocked the LTD induction at 5 min (ANOVA: $F(1,4) = 10.055$, $p = 0.034$) and at 15 min ($F(1,4) = 8.349$, $p = 0.045$) post-tetanus. To rule out the possibility that the first LTD stimulation to the SCC itself might prevent LTD of fEPSPs evoked by a second LTD stimulation (50 - 70 min interval between the two LTD stimuli), in 3 different slices we reversed the order of drug application. When we first pretreated the slices with 1 μM naloxone and gave LTD stimulation, once again we observed no LTD; but after naloxone washout for 20 - 30 min, we gave a second LTD stimulation to the same slice and now induced clear LTD of fEPSPs (data not shown).

In another set of experiments we compared the slope of fEPSPs measured from slices exposed only to control media, with those from a different set of slices preincubated for 30 min with 1 μM naloxone. In control conditions, 20 min after a strong stimulus applied to SCC fibers the initial slope of fEPSPs was depressed to 47 ± 9% of the control. However, in the presence of 1 μM naloxone, LTD stimulation was ineffective in eliciting LTD of fEPSPs: the initial slope of the fEPSPs was 100 ± 17% 5 min after LTD stimulation ($n = 8$; Fig. 4). A significant difference was observed between control and naloxone groups at 5 min ($F(1,11) = 7.413$, $p = 0.020$), at 10 min ($F(1,11) = 5.294$, $p = 0.042$), at 15 min ($F(1,11) = 10.862$, $p = 0.007$), and at 20 min ($F(1,11) = 13.846$, $p = 0.004$) post-tetanus.

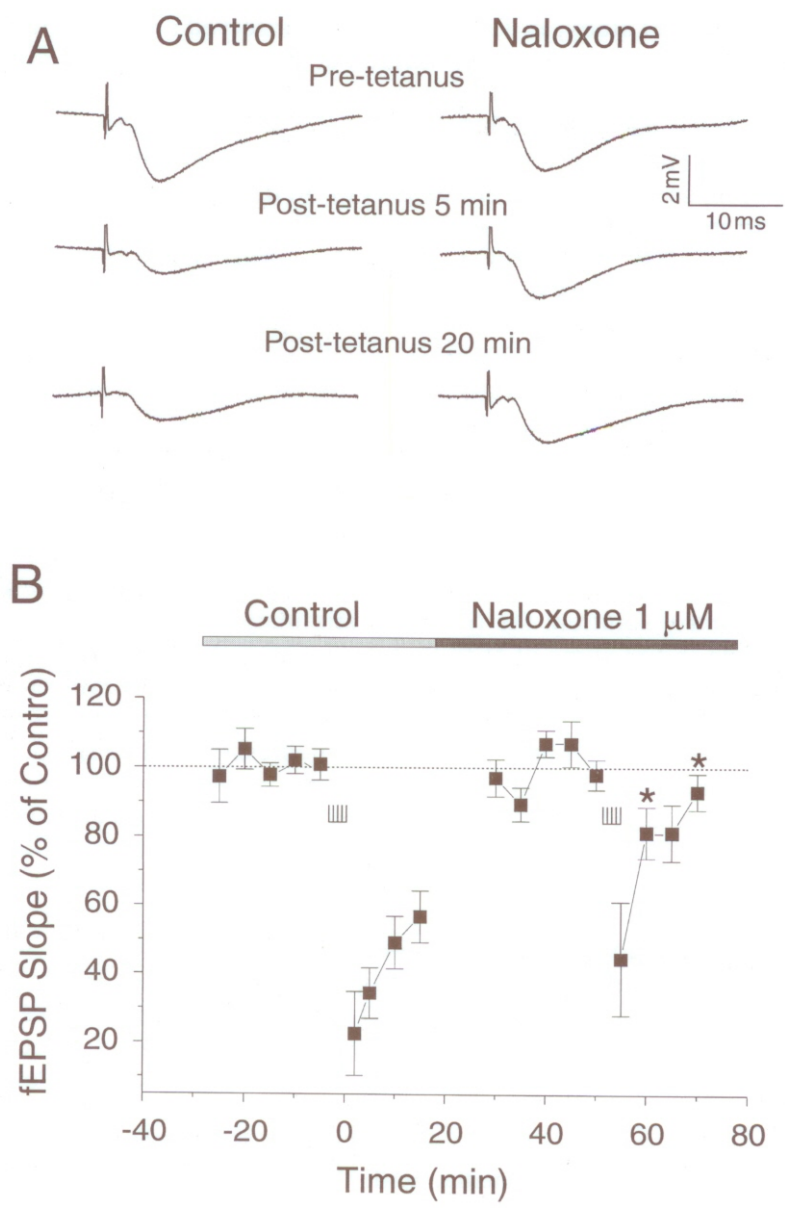

Fig. 3. - *Naloxone blocks LTD of fEPSPs.*

A: In control conditions (left panel), LTD stimulation to the SCC region induced LTD of fEPSPs at 5 and 20 mins. After 20 min post-tetanus, the stimulation intensity was increased to bring the fEPSP slope values back to control levels. In the right panel, the same slice was then treated with 1 μM naloxone for 30 min and subsequently given a second LTD stimulation. Naloxone pretreatment blocked the LTD of fEPSPs at 5 and 20 min. B: Graph of all experiments (n = 5) using the same method as above. Dashed line indicates 100% of control; symbols = tetanus trains; error bars = S.E.M.; asterisks show significant difference between control and naloxone treatments at 5 min (see Results; ANOVA; $p = 0.0338$), and at 15 min ($p = 0.0446$) post-tetanus.

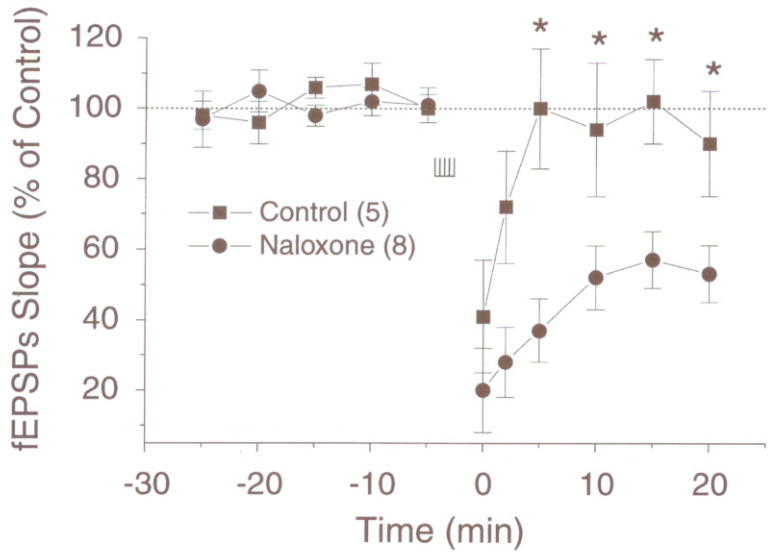

Fig. 4. - *Averaged fEPSP responses to LTD stimulation under different pharmacological conditions.*

In control ACSF, slices (n = 5) given LTD stimulation to the SCC region showed LTD of fEPSPs for greater than 20 min. In a diffetent set of slices (n = 8), pretreatment with 1 μM naloxone for 30 min blocked LTD of fEPSPs recorded at 5, 10, 15, and 20 min. Dashed line indicates 100% of control; symbol = tetanus trains; error bars = S.E.M.; asterisks show significant difference between control and naloxone groups at 5 min ($p = 0.0199$) at 10 min ($p = 0.0421$), at 15 min ($p = 0.0072$), and at 20 min ($p = 0.004$) post-tetanus.

DISCUSSION

In the present study we used strong tetanic stimulation of SCC fibers in CA1 to induce homosynaptic fEPSP-LTD persisting longer than 40 min. Naloxone blocked the induction of this LTD, suggesting that opioid peptides may mediate the cellular processes underlying the LTD. As far as we are aware, this is the first report suggesting that opioid peptides may mediate LTD in CA1.

In our initial studies, like Bashir *et al.* (2), we used tetanic stimulation only twice the test intensity and induced synaptic depression of the fEPSPs lasting only 5 - 10 min (data not shown). Therefore, in our later studies we used higher tetanic stimulation with intensities 3 - 5 times the test stimulus and evoked a long-lasting depression of fEPSPs. Barr *et al.* (1) recently reported that theta-burst stimulation at 10 times the test strength did not induce LTP but produced lasting depression of previously potentiated responses (depotentiation). In their study as well as ours, stimulation of the same pathway could elicit either LTP or depression of synaptic events, depending on the stimulus intensity. However, Barr and coworkers (1) did not observe LTD in control conditions. This discrepancy might be due to the use of different stimulus paradigms (e.g. theta vs. tetanic trains) in the two studies.

The stronger tetanus intensity used to elicit LTD in our study may release

endogenous opioids in addition to glutamate. In the hippocampus as well as in other brain regions, release of endogenous opioid peptides (or peptides in general) usually requires stronger stimulation intensities (and higher frequency trains) than required for glutamate release (38). Indeed, several studies using high frequency stimulation paradigms have demonstrated endogenous opioid peptide release in the hippocampus (10, 11, 39, 40). Both mossy fiber and PP pathway stimulation to CA3 and dentate gyrus, respectively, evoked release of endogenous opioid peptides, and these peptides have been shown to enhance or inhibit LTP in a naloxone-sensitive manner (10, 26, 39).

Most opiate studies in the CA1 region have focused on the effects of application of exogenous opioid peptides in inhibiting inhibitory interneurons. The resulting disinhibition hypothesis (see 34 for review) may derive from activation of either mu or delta subtypes of opiate receptors (9, 22, 23, 25, 32, 36, 45). However, endogenously released opioid peptides in the CA1 may have different effects than exogenous opioid application. Exogenous application, especially by the superfusion method, might be expected to reach all receptors and have additional effects from those derived from synaptic release. There are several reports indicating that opioid-containing fibers from the EC innervate the most distal dendrites of CA1 neurons (5, 27). Another study found that enkephalin-like immunoreactivity was localized within two different interneurons, one type located between stratum radiatum and stratum lacunosum moleculare and the other within the stratum radiatum of CA1 (16). Dynorphin-positive fibers are also present in the stratum radiatum and in the stratum lacunosum moleculare of CA1 (27).

Thus, it is likely that our stimulating electrodes were placed in the region that contains enkephalin and dynorphin-like immunoreactivity (see Fig. 1A). The strong stimulation intensity used to elicit LTD might be expected to release endogenous enkephalin and dynorphin. Exogenous dynorphin has been shown in the CA1 region to act through either mu or delta receptors (4, 31). However, endogenous dynorphin may also activate kappa receptors. We have previously reported that low concentrations of dynorphin (less than 1 μM) enhance the voltage-dependent K+ conductance known as the M-current in CA3 pyramidal neurons and that this effect is blocked by naloxone and the $kappa_1$ selective antagonist, norbinaltorphimine (29). After strong tetanic stimulation, release of endogenous dynorphin might also augment the M-current in CA1 neurons. Since the M-current inhibits neurons, it could play a role in LTD. Interestingly, Weisskopf *et al.* (40) have shown that release of dynorphin can inhibit the induction and expression of mossy fiber LTP. Our data showing blockade of LTD by naloxone suggest that either mu, delta or kappa opioid receptors might be involved in LTD of fEPSPs. We are currently using more subtype-specific opioid receptor antagonists to determine what subtype might be involved in LTD.

The recent study by Xie and Lewis (43) has demonstrated that tetanic stimulation of the PP elicits an LTP of inhibitory postsynaptic currents in dentate, and that endogenous opioids regulate this LTP. These authors showed that a high frequency stimulus train induced a long-lasting enhancement of a $GABA_A$ synaptic compo-

nent only in the presence of naloxone. They also suggested that this enhacement may arise from LTP of excitatory synapses of PP terminals on GABAergic interneurons. In another study, single stimuli applied to the same PP fiber area as in the present study reduced Schaffer collateral-mediated EPSPs in CA1 pyramidal cells (15). Such an inhibitory effect might be enhanced by our strong tetanus. However, intracellular recording will be required to unravel the mechanisms underlying the LTD of CA1 fEPSPs evoked strong tetanic stimulation of PP.

We used the specific NMDA receptor antagonist AP5 to determine if NMDA was involved in LTD of fEPSPs. AP5 did not block either the induction of LTD or the DC shift usually seen with strong tetanic stimulation. Recently, Paulsen *et al.* (33), described a short-term form of synaptic depression resistant to APV in CA1 region. In contrast, Bashir and Collingridge (2) reported that both the DC shift and the transient synaptic depression seen with strong tetanic stimulation were blocked in the presence of AP5. However, these authors had superfused the hippocampal slices with 50 μM of picrotoxin to eliminate $GABA_A$ inhibition. In disinhibited slices tetanic stimulation of SCC fibers may activate more NMDA receptors by eliciting a greater depolarization. Therefore, NMDA receptors could contribute to a positive feedback loop leading to a kind of spreading depression (8). Other studies using low frequency stimulation (1 - 3 Hz) have demonstrated LTD blockade by APV in the CA 1 region (14, 30).

In summary, our studies show that strong tetanic stimulation to SCC fibers can elicit LTD of fEPSPs in the CA1 region. This fEPSP-LTD is blocked by naloxone but resistent to the NMDA receptor antagonist AP5. These data are consistent with the naloxone-sensitive, NMDA receptor-independent LTPs previously descibed for the CA3 and some dentate regions (12, 44) and suggest that release of endogenous opioid peptides may mediate some forms of long-term synaptic depression. LTD has been described as a mechanism that impairs cognitive function (14). Findings from behavioral studies have suggested that some opioid peptides also impair, whereas naloxone can improve, spatial memory (17, 18, 28). Our data are consistant with naloxone improvement of spatial memory and suggest a cellular mechanism to explain opioid impairment of this function.

SUMMARY

In rat hippocampal slices, high intensity tetanic stimulation (two 1 s trains of 100 Hz separated by 20 s, 3 - 5X intensity of the test stimulus) of the Schaffer collateral-commissural (SCC) fibers induced a long-term depression (LTD) of the negative field excitatory postsynaptic potentials (fEPSP) in stratum radiatum of the CA1 region. The initial slope of the fEPSP, evoked by a single test shock applied to the SCC fibers, was depressed for a period longer than 40 min following such high intensity tetanic stimulation to this fiber system. However, the same tetanic stimulation delivered at low (test) intensity induced long-term potentiation (LTP) of the fEPSPs. Thus, similar patterns of stimulation can induce either LTP

or LTD, depending on whether low- or high-intensity tetanic stimuli are delivered. The LTD induced by high strength tetanic stimulation was clearly blocked by the opioid antagonist naloxone (1 μM); however, the N-methyl-D-aspartate (NMDA) receptor antagonist D-2-amino-5-phosphonopentanoate (AP5; 50 μM) had no effect on the LTD. Our data suggest that the strong stimulation used for LTD induction may have activated other afferent fiber systems and/or local interneurons in addition to SCC fibers, such as the enkephalin-containing terminals of the perforant path (PP) projecting to the stratum lacunosum moleculare or opioid peptide-containing interneurons. Thus, the resulting release of endogenous opioid peptides could play a role in the cellular mechanisms involved in some forms of long-term synaptic depression.

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