

# Electrophysiological and Pharmacological Characterization of the Direct Perforant Path Input to Hippocampal Area CA3

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**Berzhanskaya, Julia, Nathaniel N. Urban, and German Barrio-nuevo.** Electrophysiological and pharmacological characterization of the direct perforant path input to hippocampal area CA3. *J. Neurophysiol.* 79: 2111–2118, 1998. Monosynaptic perforant path responses evoked by subicular stimulation were recorded from CA3 pyramidal cells of rat hippocampal slices. These monosynaptic responses were isolated by using low intensities of stimulation and by placing a cut through the mossy fibers. Perforant path-evoked responses consisted of both excitatory and inhibitory components. Excitatory postsynaptic currents (EPSCs) were mediated by both  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) and *N*-methyl-D-aspartate receptors (NMDAR). Inhibitory postsynaptic currents consisted of  $\gamma$ -aminobutyric acid-A (GABA<sub>A</sub>-) and -B (GABA<sub>B</sub>-) receptor-mediated components. At membrane potentials more positive than -60 mV and at physiological  $[\text{Ca}^{2+}]/[\text{Mg}^{2+}]$  ratios, >30% of perforant path evoked EPSC was mediated by NMDARs. This value varied as a function of the membrane voltage and external  $[\text{Mg}^{2+}]$ . Two types of responses were observed after low-intensity stimulation of the perforant path. The first type of response showed paired-pulse facilitation and was reduced by 2-amino-4-phosphonobutyric acid (AP4). The second type of response showed paired-pulse depression and was reduced by baclofen. Electrophysiological and pharmacological characteristics of these two types of responses are similar to the properties of lateral and medial perforant path-evoked EPSPs in the dentate gyrus.

## INTRODUCTION

In the classical “trisynaptic circuit” model of the hippocampus, pyramidal cells of the CA3 region of the hippocampus receive their primary input from the mossy fibers (MF), the axons of the dentate granule cells. However, anatomic data show that pyramidal cells of CA3 also receive a direct, monosynaptic input from the entorhinal cortex (EC), via the fibers of the perforant path (PP) (Steward 1976). Because an almost equal number of synapses are formed by PP fibers in CA3 and in the dentate gyrus (DG) (Amaral et al. 1990), the PP projection to CA3 could be as strong as the PP projection to the DG. Findings from electrophysiological experiments in vitro (Doller and Weight 1982) and in anesthetized animals in vivo (Yeckel and Berger 1990, 1995) also suggest that the monosynaptic input from the EC to areas CA1 and CA3 is sufficiently strong enough to excite pyramidal cells in these areas to the level of action potential generation. In addition, McNaughton et al. (1989) have demonstrated that place cell firing in areas CA1 and CA3 of behaving rats persists after destruction of the DG. These data suggest that the direct PP input to areas CA1 and CA3 plays a significant role in hippocampal function. Several

computational models of the hippocampal formation already have incorporated the direct PP projection to areas CA3 (O'Reilly and McClelland 1994) and CA1 (Hasselmo and Schnell 1994) as a functional part of cortico-hippocampal connections.

Despite the recognition of the functional importance of the direct input from EC to the hippocampus proper, the pharmacological and electrophysiological properties of the monosynaptic PP input to area CA1 and, especially, to area CA3 have not yet been characterized fully. Previous studies have shown that PP-evoked excitatory postsynaptic currents (EPSPs) are accompanied by inhibitory postsynaptic currents (IPSPs) both in the DG (Buckmaster and Schwartzkroin 1995; Staley and Mody 1992) and area CA1 (Colbert and Levy 1992; Empson and Heinemann 1995a). These IPSPs are thought to be disynaptic (Empson and Heinemann 1995b); and anatomic data suggest that hippocampal interneurons receiving direct PP input mediate this disynaptic inhibition (Buhl et al. 1994; Kiss et al. 1996). However, PP input to the DG was found to be mainly excitatory, whereas in CA1, PP stimulation results in both excitation and strong inhibition (Empson and Heinemann 1995a). It has been demonstrated that inhibition can change dendritic excitability (Kim et al. 1994) and reduce EPSPs by linear or nonlinear summation (Staley and Mody 1992). The role of inhibition in the response of CA3 pyramidal cells to the PP stimulation is still unknown.

PP-evoked responses in the DG and area CA1 (Colbert and Levy 1992; Lambert and Jones 1989) were found to be mediated in part by *N*-methyl-D-aspartate (NMDA) receptors (NMDAR). The NMDAR-mediated component represents a significant part of the response (~30%) in the DG (Colino and Malenka 1993; Keller et al. 1991). Although the NMDAR-mediated component of the PP-evoked response in area CA1 has been studied (Colbert and Levy 1992; Empson and Heinemann 1995b), it has not been quantified. The presence of an NMDAR-mediated component of PP-evoked EPSPs in area CA3 has not been demonstrated directly. However, the ability to induce NMDAR-dependent long-term potentiation of the PP input to CA3 (Berger and Yeckel 1991) indicates that some portion of PP-evoked responses in this area must be NMDAR mediated at least under conditions of tetanic stimulation.

The heterogeneity of the PP input to the hippocampus adds another dimension to the characterization of this input. Specifically, the PP input to the hippocampus consists of medial and lateral PP fibers (MPP and LPP, respectively)

(Amaral 1993; McNaughton 1980; Steward 1976). Thus stimulation of PP fibers may result in combined LPP/MPP responses. In the DG, LPP and MPP form synapses in adjacent portions of *stratum moleculare*. LPP synapses are located more distally on the dendritic tree, whereas MPP synapses are located more proximally (Steward 1976; Witter 1993). A similar distribution of LPP and MPP terminals exists in area CA3. The LPP- and the MPP-evoked responses in the DG can be differentiated by their kinetics, paired-pulse profile and different sensitivity to neuromodulators (Bramham et al. 1988, 1991; Colino and Malenka 1993; Koerner and Cotman 1991; Lanthorn and Cotman 1981; McNaughton 1980). Similar differences between LPP and MPP inputs to area CA3 have been observed in vivo (Breindl et al. 1994).

Most previous studies of the PP input to CA3 were done in vivo preparations, in which contamination of the monosynaptic PP input to CA3 by polysynaptic responses is difficult to avoid. Hence, the first goal of this study was to identify conditions under which the isolated monosynaptic PP input to area CA3 could be studied using the advantages of the hippocampal slice preparation. The second goal was to determine which receptors mediate excitatory and inhibitory components of isolated PP-evoked response in area CA3. The third goal was to determine the proportion of the NMDAR-mediated component of PP-evoked EPSCs. Finally, we aimed to investigate differences between LPP- and MPP-evoked responses in area CA3.

## METHODS

### *Slice preparation and recordings*

Transverse hippocampal slices (450  $\mu\text{m}$  thick) were obtained as described previously (Henze et al. 1995; Urban and Barrionuevo 1996). In all slices, a cut was made through the hilus of the DG up to the suprapyramidal blade of dentate to prevent contamination of the monosynaptic PP response by MF. Recordings were made from slices submerged in carbogenated artificial cerebrospinal fluid [concentrations were (in mM) 125.0 NaCl, 2.0 KCl, 1.2  $\text{NaH}_2\text{PO}_4$ , 26.0  $\text{NaHCO}_3$ , 10.0 dextrose, 3.0  $\text{MgCl}_2$ , and 3.0  $\text{CaCl}_2$ ] at temperature 31–33°C and rate of perfusion 2 ml/min.

A bipolar stimulation electrode (62  $\mu\text{m}$  diam nichrome wire) was positioned in the *s. lacunosum moleculare* on the border of the subiculum and area CA1 (Fig. 1C), where myelinated PP fibers are clearly visible under the dissecting microscope. In studies of excitatory and inhibitory components, the position of stimulation electrode was close to the hippocampal fissure (350–100  $\mu\text{m}$ ) and did not vary throughout the experiment. In experiments in which we tried to isolate LPP responses from MPP responses by changing stimulation site, the stimulation electrode was moved within a range of 100  $\mu\text{m}$  perpendicular to the PP fiber tract. Field potentials for the laminar and the current source density analysis (CSD) study were recorded using glass microelectrodes (1–3 M $\Omega$ ) filled with 0.5 M NaCl solution at multiple positions within area CA3.

When collecting data for CSD, the first recording site was always in the *s. lacunosum moleculare* of CA3. While recording at this location, the stimulation intensity was adjusted to be 60% of the value required to elicit a population spike. The depth ( $z$  axis) of the recording electrode was adjusted to maximize the peak amplitude of the recorded field potential. Ten consecutive responses were recorded at this location before the electrode was moved 50  $\mu\text{m}$  toward the *s. pyramidale* ( $x$  axis). At this new location, the electrode was placed into the tissue at the same depth as in the

previous position. Every 200  $\mu\text{m}$  (i.e., every fourth position) the depth of the electrode was varied by  $\pm 50$   $\mu\text{m}$  in the  $z$  axis, and 10 responses were recorded; if responses recorded in either of these positions were larger than those observed in the original  $z$  position, the experiment was terminated and the data were not used. This procedure was continued until the recording electrode was moved  $\geq 150$  mm beyond the cell body layer, at which point it was returned to the original placement in the *s. lacunosum moleculare* and field potentials were recorded again at this position. If the average peak amplitude of these field potentials differed by  $>20\%$  of the amplitude originally recorded at this position, then the data were not used. The electrode then was moved to positions away from the cell body layer, until it reached the hippocampal fissure. These field potential data then were transformed into CSD data using the algorithm D2 described by Freeman and Nicholson (1975). Tissue conductivity was assumed to be constant and equal to 1, and therefore, CSDs are expressed in arbitrary units proportional to actual current densities.

Whole cell recordings (WCR) (Blanton et al. 1989) were made via Axopatch-1D patch-clamp amplifier (Axon Instruments). WCR pipettes (3–7 M $\Omega$ ) were prepared from borosilicate glass and filled with one of three WCR solutions (see further). Series resistances ( $<20$  M $\Omega$ ) were not compensated and were estimated as described by Langdon et al. (1995). Only traces with stable series resistance were analyzed.

Experiments were begun 30–40 min after breaking into the cell to allow for complete cell dialysis. The stimulation intensity was adjusted to elicit postsynaptic responses the amplitude of which was 25–30% of maximum amplitude. This intensity was less than or equal to the stimulation intensity used in CSD experiments. Paired pulse stimulation (ISI = 60–80 ms) was used in the beginning of all experiments. In studies of MPP/LPP responses, ISI intervals varied from 30 to 200 ms to show the persistence of paired-pulse facilitation (PPF) or paired-pulse depression (PPD). Electrophysiological responses were filtered at 3–5 kHz, digitized, and stored on a computer for off-line analysis. Either responses to single-pulse stimulation or the first response to paired-pulse stimulation were used for  $I$ - $V$  plots and NMDAR component estimation. All traces shown are averages of 6–10 individual waveforms.

### *Solutions and drug application*

WCR pipettes were filled with one of three solutions: CsF + PTX [which contained (in mM) 120 cesium fluoride, 20 CsCl, 1 bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA), 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and 0.15–0.2 picrotoxin]; Cs-gluconate [which contained (in mM) 115 cesium gluconate, 20 CsCl, 10 HEPES, 10 ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 10 sodium phosphocreatine, 4 MgATP, and 0.3 GTP plus 50 U/ml creatine phosphate]; or K-gluconate [which contained (in mM) 115 potassium gluconate, 20 KCl, 10 HEPES, 10 EGTA, 10 sodium phosphocreatine, 4 MgATP, and 0.3 GTP plus 50 U/ml creatine phosphate]. The pH and osmolality of the solutions were 7.2–7.4 and 270 mosM, respectively.

All drugs were delivered by bath application. The following drugs were used: the AMPA receptor (AMPA) antagonist, 6-cyano-7-nitroxaline-2,3-dione (CNQX; 10  $\mu\text{M}$ ), the NMDAR antagonist, D-2-amino-5-phosphovalerate (D-APV) (25  $\mu\text{M}$ ), the GABA<sub>A</sub> antagonist bicuculline methiodide (10  $\mu\text{M}$ ), and the GABA<sub>B</sub> antagonist CGP35348 (500  $\mu\text{M}$ ). The GABA<sub>B</sub> agonist baclofen was applied in concentrations of 2–5  $\mu\text{M}$ , the mGluR agonist (D,L)-2-amino-4-phosphonobutyric acid (AP4) in concentrations of 25–50  $\mu\text{M}$  to affect selectively either MPP- or LPP-evoked responses (Koerner and Cotman 1991; Lanthorn and Cotman 1981). All drugs were purchased from Sigma (St. Louis, MO)

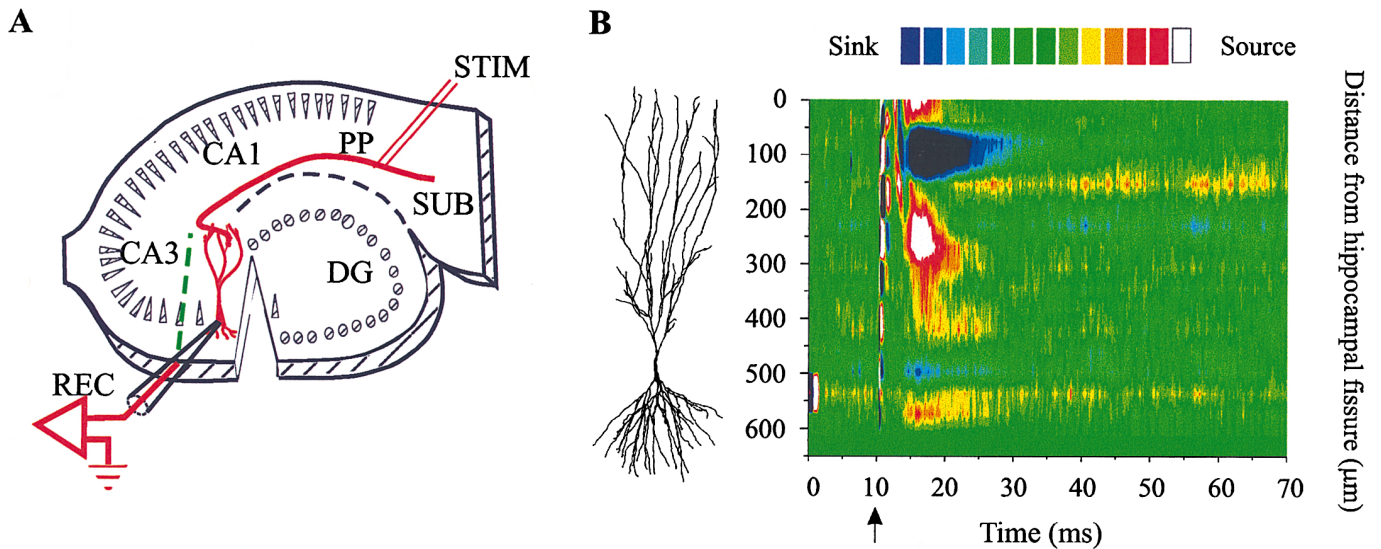


FIG. 1. Isolation of the perforant path projection to CA3. *A*: scheme of a hippocampal slice preparation showing stimulation (STIM) and recording (REC) sites and placement of cut. Recordings were made from CA3 pyramidal cells located in CA3b and CA3c subfields. Extracellular electrode in the CSD study was moved in the direction perpendicular to the cell bodies layer (green line). *B*: Current source density profile within CA3 indicates selective activation of perforant path synapses. Current source density analysis was calculated from field potentials recorded in CA3 after stimulation of the perforant path as described in METHODS. Contour plots show that the main current sink is located in *stratum lacunosum moleculare* with very little activation elsewhere on the dendritic tree of CA3 pyramidal cells.

with exception of D-APV (Research Biochemicals, Natick, MA) and CGP (generous gift of Giba Geigy, Basel, SZ).

## RESULTS

### Isolation of monosynaptic PP-evoked response

Electric stimulation in the subiculum can result in multiple polysynaptic responses due to activation of intrinsic hippocampal circuits. Specifically, the monosynaptic PP response in area CA3 can be contaminated by disynaptic responses due to activation of MFs, disynaptic responses due to activation of collaterals of CA3 pyramidal cells, and direct recruitment of Schaffer collaterals in area CA1 resulting in either antidromic activation of CA3 pyramidal cells or monosynaptic responses due to activation of collateral branches in this area. To eliminate responses from mossy fibers, we placed a cut between the DG and area CA3 up to the suprapyramidal blade of dentate (Fig. 1*A*). In addition, we placed the stimulation electrodes far from area CA3 (Fig. 1*A*), as close as possible to the hippocampal fissure, and used low stimulation intensities that resulted in responses with amplitude <30% of maximum amplitude. These measures were taken to reduce the probability of antidromic stimulation of CA3 pyramidal cells, and of activation of CA3 collaterals. Absence of collateral and antidromic activation was verified by the lack of an evoked population spike in s.pyramidale as measured extracellularly (data not shown) and by the distribution of current sinks and sources, as determined by CSD analysis (Fig. 1*B*).

Data for CSD analysis that met the criteria described in METHODS were collected from five slices. The strength of stimulation was <60% of that required to elicit a population spike. All CSDs showed a pattern of sources and sinks that is similar to the example in Fig. 1*B*. PP stimulation resulted

in a large, short latency current sink, of <10 ms duration, in an area between 50 and 150 μm from the hippocampal fissure. This large current sink was accompanied by a current source in the *s. radiatum* and was followed a few milliseconds later by a small current source in the CA3 cell body layer. No evidence was seen for current sinks of significant amplitude in either the *s. radiatum* or the cell body layer (for comparison, a small current negativity at 15–20 ms and at 500 μm from the hippocampal fissure represents <3% of the *s. lacunosum moleculare* current sink). This indicates that stimulation in *s. lacunosum moleculare* of the subiculum resulted in specific activation of PP synapses.

To assess the validity of the assumption that all current sinks and sources were located in recording area, the sum of current sinks and sources was calculated for all time points during two time periods: 3–8 ms before PP stimulation and 12–60 ms after PP stimulation. This sum averaged  $-0.45 \pm 33$  units/time point (mean  $\pm$  SD) before stimulation and  $0.58 \pm 27$  units/time point (SD) after stimulation. Maximal sum of sinks and sources after stimulation was 39 units/time point, which is within 1.2 SD from the sum value before stimulation.

We concluded that by using low intensities of stimulation, positioning the stimulation electrode far from area CA3 and making a cut as described above, we were able to isolate the monosynaptic PP-evoked responses for the following pharmacological analysis.

### Isolation of EPSC and IPSC evoked by PP stimulation

Stimulation of the PP resulted in a composite response in CA3 pyramidal cells that consisted of both inward and outward currents (Fig. 2*C*) when recorded in the whole cell mode at  $-80$  mV. To isolate EPSCs and different compo-



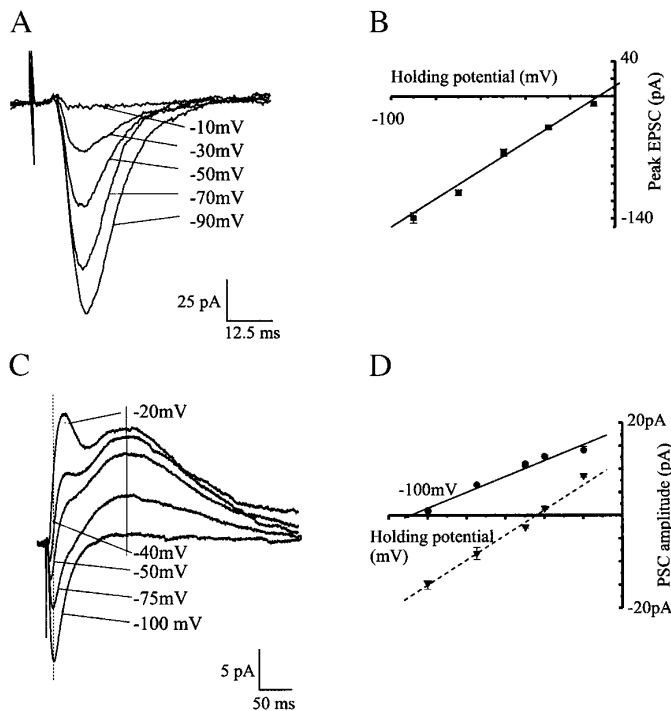


FIG. 2. *I-V* relations for perforant path-evoked responses. *A*: excitatory postsynaptic currents (EPSCs) measured at various holding potentials (CsF + picrotoxin in the whole cell recordings pipette). *B*: plot of EPSC peak amplitude (at 15 ms from the stimulation artifact) vs. holding potential. *C*: perforant path-evoked responses recorded with a pipette containing K-gluconate had 3 components: early (peak at 6 ms, reversal at  $-20$  mV), intermediate (peak at 20 ms, reversal at  $-43$  mV), and late (peak at 130 ms, reversal at  $-100$  mV). *D*: *I-V* plot for the late and the intermediate components.

nents of IPSCs, we varied the contents of the recording pipette. Both fluoride ( $F^-$ ) and picrotoxin block  $GABA_A$   $Cl^-$  channels when applied internally (Bormann 1988; Inomata et al. 1988; Kay 1992), and Cs blocks  $GABA_B$   $K^+$  channels (Gahwiler and Brown 1985). We therefore used CsF + picrotoxin WCR solution to isolate EPSCs (Figs. 2*A* and 4). Alternatively, we used Cs-gluconate WCR solution to suppress the  $GABA_B$ -mediated component of IPSC and to leave the  $GABA_A$ -mediated component intact (Fig. 3, *A* and *B*). Finally, we used K-gluconate WCR solution, which supports glutamatergic and both  $GABA_A$  and  $GABA_B$  conductances (Steilzer et al. 1988), to study the intact PP-evoked response (Figs. 2*C* and 3*C*).

The reversal potential of isolated EPSCs in a sample cell was  $-8.4$  mV (Fig. 2*B*; mean reversal across experiments was  $-3.1 \pm 6.2$  mV (SD,  $n = 11$ ). These EPSCs were suppressed

partially by addition of the NMDAR antagonist D-APV (25 mM) to the bath solution (Fig. 4*B*) and were suppressed completely in the presence of both D-APV and CNQX. These results indicate that PP-evoked EPSCs are glutamatergic and mediated by both AMPARs and NMDARs.

With Cs-gluconate in the pipette the PP-evoked response reversed at about  $-40$  mV. This reversal potential indicates that the response consists of both an EPSC and a  $GABA_A$ -mediated IPSC. We isolated pure  $GABA_A$ -mediated IPSCs by recording at  $-10$  mV (close to the EPSC reversal potential; Fig. 3*A*). These IPSCs were suppressed completely by the addition of bicuculline (10  $\mu$ M). However, EPSCs and IPSCs did not add linearly. Thus at the reversal potential of the  $GABA_A$ -mediated IPSCs ( $-50$  mV), application of bicuculline resulted in an increase of the amplitude of the depolarizing response (Fig. 3*B*). This indicates that in control conditions EPSCs are reduced by  $GABA_A$ -ergic shunting (50% reduction at  $-50$ – $-60$  mV).

Finally, all three components of the PP-evoked response, excitatory, fast and slow inhibitory, were observed with K-gluconate in the WCR pipette (Fig. 2, *C* and *D*). The intermediate,  $GABA_A$ -ergic component, as in the previous set of experiments, reversed at about  $-40$  mV. The long-lasting component of the PP-evoked response reversed at  $-100$  mV and was suppressed in the presence of the  $GABA_B$  antagonist, CGP35348 (500  $\mu$ M; Fig. 3*C*). On the basis of these data, we conclude that the long-lasting inhibitory component is mediated by  $GABA_B$  receptor-dependent conductances.

#### Proportion of the NMDAR-mediated component of PP-evoked EPSC

EPSCs isolated with CsF + picrotoxin WCR solution were analyzed to provide with a quantitative estimate of the proportion of the evoked EPSC that is mediated by NMDARs. With the stimulation paradigm described above, only responses showing PPF were recorded and are analyzed in this section. NMDAR-mediated EPSC (NMDAR-EPSC) component was isolated by applying CNQX (10  $\mu$ M; Fig. 4*A*). In the absence of AMPAR-EPSCs, NMDAR component was strongly reduced, therefore the intensity of stimulation was increased to obtain a measurable response. In other experiments, NMDAR-mediated EPSCs were obtained by subtraction of EPSCs recorded in the presence of D-APV (25  $\mu$ M) from control EPSCs (control-APV, Fig. 4*B*). The ratio of the peak amplitude of NMDAR-EPSC to the peak amplitude of control EPSCs was used to determine the contribution of NMDAR-EPSCs to the total EPSC at different membrane potentials, and different extracellular  $[Mg^{2+}]$ . The integral

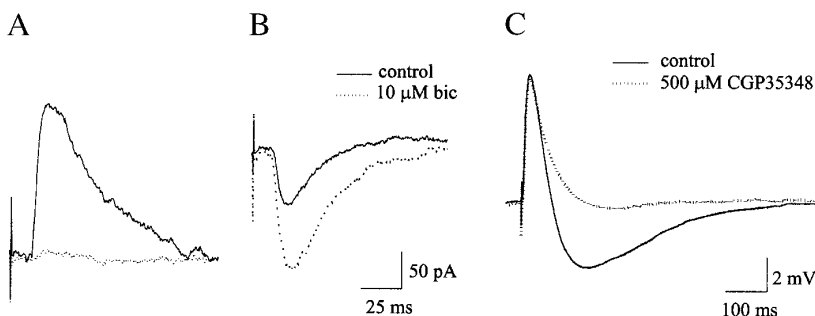


FIG. 3. Perforant path-evoked inhibitory responses. Mixed EPSC with  $\gamma$ -aminobutyric acid- $A$   $GABA_A$ -mediated inhibitory postsynaptic current (IPSC; control), and isolated EPSC after suppression of IPSC with bicuculline at  $-10$  mV (*A*) and  $-50$  mV (*B*) (pipette solution contained Cs-gluconate). *C*: perforant path-evoked response recorded in current-clamp using pipette containing K-gluconate. Long-lasting inhibitory component was suppressed by addition of CGP 35348 (500 mM).

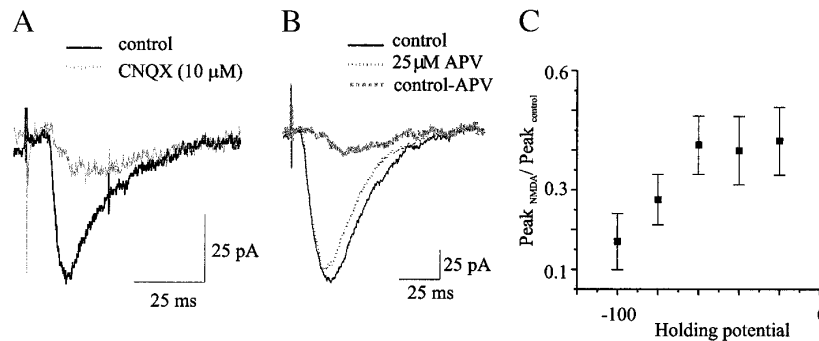


FIG. 4. Pharmacological isolation of excitatory components of the perforant path-evoked EPSC. *A*: control recordings ( $[Mg^{2+}] = 3$  mM, holding potential,  $-60$  mV) and *N*-methyl-D-aspartate receptor (NMDAR)-mediated component of EPSC isolated in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX;  $10$  mM) (holding potential,  $-60$  mV,  $[Mg^{2+}] = 1.25$  mM, stimulation intensity increased by  $50\%$ ). *B*:  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA)-mediated component of EPSC isolated by blocking NMDAR-mediated component with 2-amino-5-phosphovalerate (APV;  $25$  mM). NMDAR-mediated component calculated by subtracting AMPA-mediated component from the control response (control-APV) [holding potential,  $-80$  mV, ( $[Mg^{2+}] = 3$  mM)]. *C*: Proportion of EPSC mediated by NMDARs, calculated as the ratio of the peak amplitude of the NMDAR-mediated EPSC to the peak amplitude of the control EPSC in experiments with APV application as a function of holding potential ( $[Mg^{2+}] = 3$  mM, mean  $\pm$  SE,  $n = 6$  for each holding potential except for  $-100$  mV where  $n = 3$ ). In all experiments, the pipette solution contained CsF + picrotoxin.

of the EPSC over time (charge) is another quantitative measure for NMDAR-mediated EPSC. We calculated both the ratio of the amplitude and ratio of the charge of NMDA to control responses for five cells and found no significant differences, therefore here we report amplitude ratios only.

The amplitude of the NMDAR-mediated response varied as a function of holding potential (Fig. 4C). The NMDAR-mediated EPSC was  $40 \pm 8\%$  (SE) of the total response at  $-40$  mV and did not differ significantly from this value in a range of potentials between  $-60$  and  $-20$  mV and for both  $[Mg^{2+}]$  used ( $1.25$  and  $3$  mM). At more negative potentials, the proportion of the NMDAR-mediated EPSC decreased (Fig. 4C).

#### Variability of the paired-pulse profiles of PP-evoked EPSCs

The PP input to area CA3 consists of both LPP and MPP fibers. LPP-evoked responses in the DG show PPF of EPSPs and sensitivity to the mGluR agonist AP4 (Koerner and Cotman 1981), whereas MPP-evoked responses show PPD and are suppressed by the GABA<sub>B</sub> agonist baclofen (Lanthorn and Cotman 1981). This suppression of synaptic transmission is thought to be caused by activation of presynaptic receptors (Harris and Cotman 1985) located on the PP synaptic terminals. PP inputs to both DG and area CA3 are thought to arise from the same cell population in the EC (Tamamaki and Nojo 1993; Witter et al. 1989) and thus may show the same presynaptic properties. Data from *in vivo* studies suggest that MPP- and LPP-evoked responses in area CA3 may show the same paired-pulse profiles as those recorded in the DG (Briendl et al. 1994) (Fig. 2). We therefore hypothesized that MPP- and LPP-evoked responses in area CA3 in the slice preparation can be distinguished on the basis of their paired-pulse profiles.

With low stimulation intensities (which resulted in responses of  $<15\%$  of maximal amplitude), in a number of experiments, we observed PP-evoked EPSCs that exhibited either PPD or PPF (PPD or PPF responses, respectively; Fig. 5, A and B). We hypothesized that MPP fibers were activated selectively in experiments where PPD was evoked

by low-intensity stimulation, whereas predominant stimulation of LPP fibers was accompanied by PPF. To test this hypothesis, we applied drugs that are known to affect selectively either MPP- or LPP-evoked responses in the DG. Consistent with observations in the DG, baclofen suppressed PPD responses by  $66\%$  (measured as ratio of the amplitude of the first EPSC in the presence of drug to the amplitude of control EPSC), whereas PPF responses were reduced by only  $20\%$  (Fig. 5, A and C). On the other hand, AP4 suppressed PPF responses by  $49\%$  and PPD responses by  $6\%$  (Fig. 5, B and C). On the basis of these data, we conclude that PP-evoked EPSCs that exhibit PPD mainly represent MPP-evoked EPSCs, whereas those that exhibit PPF mainly represent LPP-evoked EPSCs.

Increasing the stimulation intensity had a similar effect

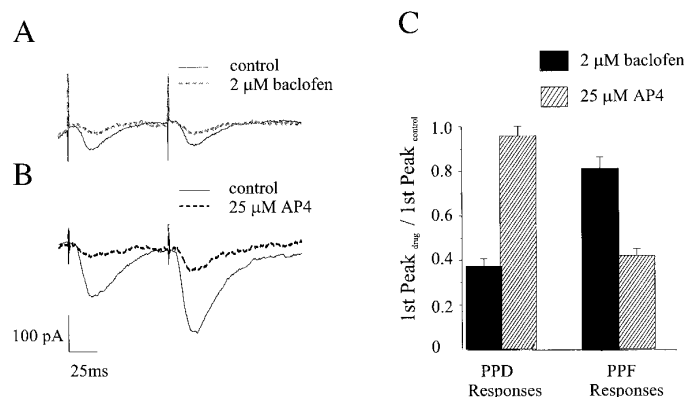


FIG. 5. Two types of perforant path responses. *A*: responses of the 1st type demonstrated paired-pulse depression in control conditions (PPD responses). Addition of baclofen suppressed PPD responses by  $65\%$ . *B*: responses of the 2nd type demonstrated paired-pulse facilitation (PPF responses). Addition of AP4 suppressed responses by  $50\%$ . *C*: summary of baclofen and AP4 action on PPD and PPF responses. Suppression of the response was determined from a ratio of the amplitude of the 1st EPSC in the pair recorded in a presence of drug (either AP4 or baclofen) to the EPSC amplitude recorded under control conditions (mean  $\pm$  SE, for PPF/AP4 experiments,  $n = 8$ ; for other experiments,  $n = 4$ ). In all experiments, the pipette solution contained CsF + picrotoxin.

on PPF and PPD responses. Specifically, it decreased PPF in responses that initially showed PPF and decreased PPD in the responses that initially showed PPD. This suggests that positioning of stimulating electrode did not result in selective activation of either pathway but instead resulted in mixed responses. In agreement with this conclusion, selectivity of drug action also was reduced when higher stimulation intensity was used. We concluded that stimulation intensity (30% of maximal) used in the pharmacological characterization of EPSCs and IPSCs reported above resulted in mixed LPP/MPP response in area CA3.

In the DG, it is possible to stimulate selectively LPP or MPP by placing the stimulating electrode in either the outer or the middle part of *s. moleculare*. We tested whether repositioning of the stimulation electrode further from the hippocampal fissure in the subiculum could result in stimulation of MPP instead of LPP. In our experiments, such change of position resulted in the shift from PPF to PPD of the whole cell measured EPSCs only in a few cases. Changes in stimulation protocol were not effective in isolating MPP- and LPP-evoked responses.

## DISCUSSION

This study is the first characterization of some of the pharmacological properties of PP-evoked monosynaptic EPSCs and disynaptic IPSCs recorded from CA3 pyramidal cells. With the hippocampal slice preparation, we isolated monosynaptic PP-evoked EPSCs and demonstrated that they are mediated by both AMPARs and NMDARs. We have shown that stimulation of the PP also results in disynaptic IPSCs, which have both GABA<sub>A</sub> and GABA<sub>B</sub> receptor-mediated components. Finally, in a number of experiments, the stimulation of the PP resulted in two electrophysiologically and pharmacologically distinct types of responses, which we identified as originating from LPP and MPP.

Our estimates of the proportion of the NMDAR-mediated EPSC are close to those reported by Keller et al. (1991) and Colino and Malenka (1993) on PP-evoked EPSCs and EPSPs components in the DG. In contrast to these findings, however, in our experiments, NMDA-mediated component began to decrease only at holding potentials more negative than  $-60$  mV. There are three possible explanations for the apparent difference in voltage dependence for the NMDAR-mediated proportion of EPSC. First, as suggested by Spruston et al. (1993), the quality of the dynamic voltage clamp may not be sufficient to prevent voltage escape from distal dendrites during synaptic events. Thus at the peak of the EPSC, the dendritic membrane potential is more positive than the holding potential at the soma and NMDA channels can be relieved from the Mg<sup>2+</sup> block at holding potentials more negative than  $-40$  mV. Consistent with this explanation NMDAR component of EPSC at  $-60$  mV was large when AMPAR-EPSCs were activated simultaneously (Fig. 4B), but it was reduced when AMPAR-EPSCs were blocked. Second, the large ratio of NMDAR- to AMPAR-mediated responses relative to that previously reported in the DG at negative membrane potentials (Colino and Malenka 1993; Keller et al. 1991) may be due to differences in electronic length between pyramidal and granule cells. Because of dendritic cable filtering, fast EPSCs (AMPAR-

mediated) arriving distally with respect to the soma (as in the case of pyramidal cells) are more attenuated than those arriving proximally (as in the case of granule cells). The difference in attenuation of the slow (NMDAR-mediated) component between pyramidal and granule cells is relatively smaller; therefore the ratio of NMDAR- to AMPAR-mediated components in pyramidal cells can be larger than that in granule cells. Finally, this difference also may represent an actual difference in the proportion of NMDA and AMPA receptors activated at PP-CA3 (compared with PP-DG) synapses.

Activation of voltage-dependent conductances, which exist in the dendrites of pyramidal cells (Magee and Johnston 1995; Stuart and Sakmann 1995) could confound the effects of drug application and distort the *I-V* function for PP-evoked responses (Bernander et al. 1994; Taylor et al. 1995). To minimize the activation of voltage-dependent conductances, we used low-intensity PP stimulation resulting in EPSCs the amplitude of which was  $<30\%$  of maximum. EPSCs evoked with this stimulation corresponded to EPSPs  $<3$  mV (measured in current clamp). EPSPs of such small amplitude have been shown to involve active dendritic conductances to only a small degree (Stuart and Sakmann 1995). In addition, CsF + picrotoxin WCR solution, which was used in our EPSC studies inactivates some Ca<sup>2+</sup> voltage-dependent conductances and blocks K<sup>+</sup> voltage-dependent conductances. Linearity of the *I-V* plot for isolated AMPA-EPSCs (not shown) also indicates that voltage-dependent conductances were not activated in these experiments.

Stimulation of the PP resulted in GABA<sub>A</sub> and GABA<sub>B</sub> receptor-mediated IPSCs in area CA3. Our data are in agreement with other reports on fast and slow IPSP components in PP-evoked responses recorded from granule cells in DG and pyramidal cells in areas CA3 (Buckmaster and Schwartzkroin 1995) and CA1 (Empson and Heinemann 1995b). We also observed nonlinear summation of EPSCs and GABA<sub>A</sub>-mediated IPSCs at negative membrane potentials, which suggests that GABA<sub>A</sub> inhibition shunts excitation evoked by PP stimulation. However, even with GABA<sub>A</sub> inhibition intact, EPSC are reduced only by 50% at membrane potentials near rest.

The PP is heterogeneous fiber tract; both LPP and MPP fibers synapse in *s. lacunosum moleculare* of area CA3. In the DG, it is possible to distinguish between LPP- and MPP-evoked responses by placement of the stimulating electrode (in the either outer or middle *s. moleculare*, respectively), kinetics (MPP responses have a faster rising phase), or paired-pulse stimulation (PPD vs. PPF for MPP or LPP, respectively). The same laminar distribution of MPP and LPP exists in CA3 area. Because the PP fibers to CA3 and DG originate from the same cell population in the entorhinal cortex, we expect that the PP-CA3 synapses will have presynaptic properties (including paired pulse facilitation/depression) similar to those observed for the PP synapses in the DG. In the present study, the type of the paired-pulse response recorded from CA3 pyramidal cells did not vary with the distance of the stimulation electrode from the hippocampal fissure. This is in agreement with anatomic studies (Steward 1976), which have demonstrated that in area CA1 and the subiculum, fibers of both the MPP and LPP are



present throughout all of the *s. lacunosum moleculare* without clear separation. However, despite the anatomic overlap of these two pathways, using very low intensities of stimulation we could observe responses that show either PPF or PPD.

We also attempted to exclude the postsynaptic factors that can affect paired-pulse ratio of PP-evoked responses. The observed differences in the paired-pulse ratio can not be accounted for by recruitment of inhibitory responses, because they were blocked by the whole cell solution. Further in LPP/MPP separation experiments, stimulation intensity was very low, which reduced the probability of antidromic or collateral contamination of EPSCs. Finally, we have compared these two types of responses with the LPP- and MPP-evoked responses observed in DG with respect to their pharmacological sensitivity. Results of differential action of baclofen and AP4 on PPD and PPF responses (Fig. 5) are in agreement with the data on selective action of these compounds on MPP and LPP synapses in the DG. Therefore, we conclude that PPD responses, which we elicited with this low intensity of stimulation, were mainly (but not exclusively) due to activation of MPP fibers, whereas PPF responses were mainly due to activation of LPP fibers. Consistent with this interpretation, the degree of PPD (for PPD responses) or PPF (for PPF responses) decreased when stimulation strength was increased to the level used in other experiments described here. This indicates that our regular stimulation protocol resulted in activation of both pathways.

In this study, we observed that PP-evoked response recorded from CA3 pyramidal cells consists of four components: AMPAR- and NMDAR-mediated excitatory and GABA<sub>A</sub>- and GABA<sub>B</sub>-mediated inhibitory ones. The NMDAR-mediated component of PP EPSP may play significant role in activity-dependent synaptic plasticity and thus will be important in the further characterization of this pathway. As we demonstrated, PP-evoked inhibition can modulate EPSPs in CA3 pyramidal cells. More detailed studies are necessary to determine whether this effect depends on strength of PP stimulation and how it may affect synaptic plasticity in this pathway.

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