

Blocking GABA_A Inhibition Reveals AMPA- and NMDA-Receptor-Mediated Polysynaptic Responses in the CA1 Region of the Rat Hippocampus

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Crépel, V., R. Khazipov, and Y. Ben-Ari. Blocking GABA_A inhibition reveals AMPA- and NMDA-receptor-mediated polysynaptic responses in the CA1 region of the rat hippocampus. *J. Neurophysiol.* 77: 2071–2082, 1997. We have investigated the conditions required to evoke polysynaptic responses in the isolated CA1 region of hippocampal slices from Wistar adult rats. Experiments were performed with extracellular and whole cell recording techniques. In the presence of bicuculline (10 μM), 6-cyano-7-nitroquinoxaline-2-3-dione (10 μM), glycine (10 μM), and a low external concentration of Mg²⁺ (0.3 mM), electrical stimulation of the Schaffer collaterals/commissural pathway evoked graded N-methyl-D-aspartate (NMDA)-receptor-mediated late field potentials in the stratum radiatum of the CA1 region. These responses were generated via polysynaptic connections because their latency varied strongly and inversely with the stimulation intensity and they were abolished by a high concentration of divalent cations (7 mM Ca²⁺). These responses likely were driven by local collateral branches of CA1 pyramidal cell axons because focal application of tetrodotoxin (30 μM) in the stratum oriens strongly reduced the late synaptic component and antidromic stimulation of CA1 pyramidal cells could evoke the polysynaptic response. Current-source density analysis suggested that the polysynaptic response was generated along the proximal part of the apical dendrites of CA1 pyramidal cells (50–150 μm below the pyramidal cell layer in the stratum radiatum). In physiological concentration of Mg²⁺ (1.3 mM), the pharmacologically isolated NMDA-receptor-mediated polysynaptic response was abolished. In control artificial cerebrospinal fluid (with physiological concentration of Mg²⁺), bicuculline (10 μM) generated a graded polysynaptic response. Under these conditions, this response was mediated both by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/NMDA receptors. In the presence of D-2-amino-5-phosphonovalerate (50 μM), the polysynaptic response could be mediated by AMPA receptors, although less efficiently. In conclusion, suppression of γ-aminobutyric acid-A inhibition reveals glutamate receptor-mediated network-driven events in the isolated CA1 region. These polysynaptic responses are mediated by AMPA and/or NMDA receptors depending on the pharmacological conditions and the external concentration of Mg²⁺ used. We suggest that these responses are driven by local recurrent collaterals of CA1 pyramidal cells.

INTRODUCTION

Recurrent excitatory connections among principal glutamatergic neurons are an important substrate for synchronization of the activity of cortical networks. In the CA3 region of hippocampus, several studies show that recurrent synapses drive polysynaptic responses after removal of GABAergic inhibition (Ben-Ari and Gho 1988; Johnston and Brown

1984; Miles and Wong 1987). In the CA1 region, several observations suggest that a similar activity may develop under certain conditions. First, although less developed than in the CA3 region, excitatory recurrent connections between pyramidal cells have been described (Deuchars and Thomson 1996; Thomson and Radpour 1991). Second, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)- and/or N-methyl-D-aspartate (NMDA)-receptor-mediated burst have been recorded after blockade of γ-aminobutyric acid-A (GABA_A) (Dingledine et al. 1986; Williamson and Wheal 1992) or adenosine A₁ receptors (in low external Mg²⁺ conditions) (Klishin et al. 1995). However, it remained unclear if these events are polysynaptic and if they are generated in the CA1 local circuit or if they propagate from CA3 via Schaffer collaterals (Wong and Traub 1983).

In a preliminary report (Crépel and Ben-Ari 1996), we showed that, in the isolated CA1 region and in low external concentration of Mg²⁺ (0.3 mM), pharmacologically isolated NMDA-receptor-mediated field potentials included a late polysynaptic component after removal of GABA_A inhibition. In the present study, we have tested the conditions required to evoke this polysynaptic response. We show that, when the NMDA-receptor-mediated response is isolated pharmacologically by addition of 6-cyano-7-nitroquinoxaline-2-3-dione (CNQX), the polysynaptic response can be evoked only in low external concentration of Mg²⁺ (0.3 mM). In contrast, if AMPA receptors are not blocked, the polysynaptic response can be evoked in physiological concentration of Mg²⁺ (1.3 mM). Under these conditions, the polysynaptic response is mediated both by AMPA and NMDA receptors. We report that, in contrast to the CA3 region, this polysynaptic response is graded and do not occur spontaneously, presumably reflecting the low level of interconnectivity between CA1 pyramidal cells (Deuchars and Thomson 1996; Thomson and Radpour 1991) and a low level of intrinsic spontaneous activity of these cells (Prince 1983). We also provide evidence that suggests that these responses are driven by local recurrent excitatory synapses.

METHODS

Slice preparation

Hippocampal slices, 450–500 μm thick, were prepared from adult male Wistar rats (3- to 4-week old) and maintained as previously described (Ben-Ari 1990). The CA1 region was surgically

isolated from the CA3 region by a knife cut. Individual slices were transferred to a recording chamber, fully submerged, and superfused at 2.5–3 ml/min (32–33°C) with artificial cerebrospinal fluid (ACSF) containing (in mM) 126 NaCl, 3.5 KCl, 2 CaCl₂, 0.3 or 1.3 MgCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃, and 10 glucose at pH 7.4 when equilibrated with 95% O₂-5% CO₂. In two sets of experiments, the concentration of divalent cations was increased to reduce strongly polysynaptic activity (Berry and Pentreath 1976; Miles and Wong 1987). High-divalent-cation ACSF was obtained by increasing the concentration of Ca²⁺ to 7 mM or by increasing the concentration of Mg²⁺ and Ca²⁺ to 6 and 4 mM, respectively (in the absence of NaH₂PO₄, thus avoiding precipitation of Ca²⁺).

Recording procedures

Responses were evoked either by orthodromic stimulation of the Schaffer collaterals/commissural pathway (15–50 μs, 25–50 V, and 0.05 Hz) or by antidromic stimulation of CA1 pyramidal cells (20–60 μs, 40–70 V, and 0.05 Hz) with a bipolar NiCr insulated wire electrode, placed in the stratum radiatum or in the alveus of the CA1 region, respectively (see Fig. 1). Extracellular recordings were performed in the stratum oriens, pyramidale, radiatum, or lacunosum moleculare, using 3 M NaCl-filled glass microelectrodes with a resistance of 2–3 MΩ. Whole cell recordings were

made in the pyramidal cell layer with glass microelectrodes having a resistance of 5–10 MΩ. The internal pipette solution contained (in mM) 140 Csfluoride, 10 NaCl, 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 10 ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 CaCl₂, 2 MgCl₂, and 1 *N*-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium bromide (QX-314), pH 7.25. Cs⁺ was used to inhibit K⁺ currents and thus improve the space clamp, and fluoride to reduce voltage-dependent Ca²⁺ currents (Kay et al. 1986). Membrane potential (*V_m*) was estimated from the potential observed upon withdrawal of the electrode from the cell. Whole cell recordings were performed using an Axopatch amplifier (Axon Instruments).

Data analysis

Responses were displayed on a Nicolet oscilloscope, a chart recorder (Gould, model 2007), and a PC computer. The recordings were digitized, stored, and analyzed using Acquis Software (G. Sadoc, Paris, France). Synaptic responses were characterized by their latency (time between the shock artifact and the onset of synaptic responses), their initial slope (measured within 3 ms from onset of the synaptic responses), their maximal amplitude, their half-maximal amplitude duration, and the time to peak response (time between the onset and the maximal amplitude of the synaptic responses). In whole cell recordings, synaptic responses were characterized by their rise time (10–90%) and decay time. To normalize synaptic responses, the amplitude of the early phase, measured at 3–5 ms after onset of the synaptic responses, was taken as reference; at this latency, the responses should be purely monosynaptic (Miles and Wong 1987). Current-source density (CSD) analysis was also performed by moving the extracellular recording electrode along a track perpendicular to stratum pyramidale, at intervals of 50 μm, from lacunosum moleculare to oriens. At each position, the electrode was lowered into the slice to a depth of 150 μm. The one-dimensional CSD at point *x* was calculated using the following formula: $I_x = (F_{x-h} - 2F_x + F_{x+h})/4h^2$, where I_x = the current at location *x*, *h* = the sampling distance (*h* = 50 μm), F_x = the field potential at location *x*, F_{x-h} = the field potential at location *x* - *h*, and F_{x+h} = the field potential at location *x* + *h* (Taube and Schwartzkroin 1988). Data are presented as means ± SE, and statistical significance was assessed using the Student's paired *t*-test analysis. The differences were considered significant when *P* ≤ 0.05.

Solutions and drugs

The NMDA-receptor (NMDA_R)-mediated response was pharmacologically isolated in the presence of 10 μM CNQX, to block AMPA receptors (AMPA_R), and 10 μM glycine (to saturate the glycine allosteric site of the NMDA_R and prevent inhibitory actions of CNQX) (Kessler et al. 1989). The AMPA_R-mediated responses were isolated pharmacologically in the presence of 50 μM D-2-amino-phosphonovalerate (D-APV, to block NMDA_R). The GABA receptors (GABA_R) were blocked by 10 μM bicuculline. Drugs were dissolved in ACSF and either were bath applied through a three-way tap system or focally applied from a micropipette by pressure (Picospritzer II, General Valve; pulses 50–500 ms duration, 2–4 bars). D-APV, CNQX, and bicuculline were purchased from Tocris Neuramin and glycine and tetrodotoxin (TTX) from Sigma.

RESULTS

The present study is based on extracellular and whole cell recordings obtained from CA1 pyramidal neurons of rat hippocampal slices. In patch clamp, within 3–5 min after

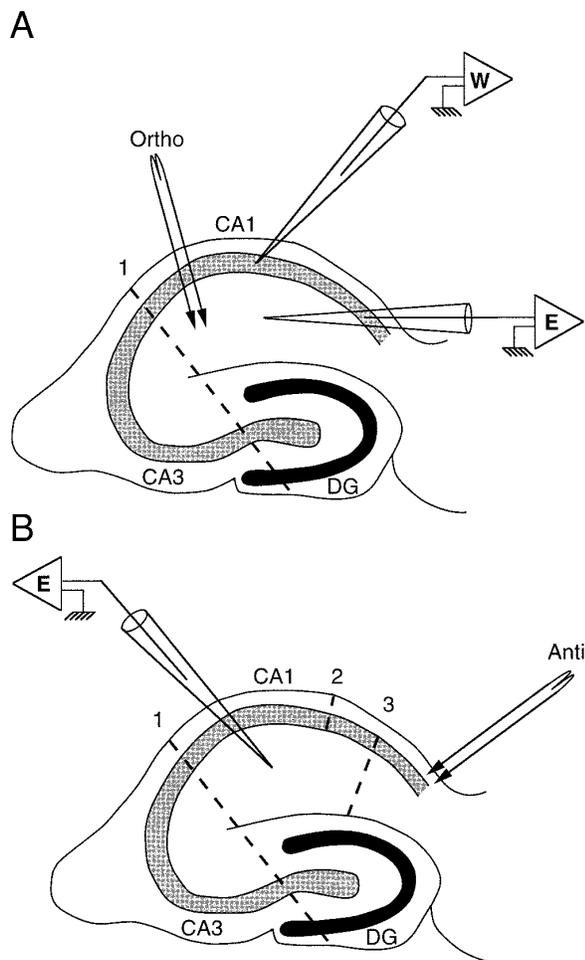


FIG. 1. Schematic drawing of hippocampal slices showing the experimental design to evoke synaptic responses by orthodromic (Ortho, A) or antidromic (Anti, B) stimulation. Dashed lines show where slices were cut to surgically isolate CA1 from CA3 (1, A and B) or to have a pure antidromic response (2 and 3, B).

the passage to whole cell configuration, CA1 pyramidal cells had a mean resting membrane potential of -64 ± 2.5 mV ($n = 35$) and a mean membrane input resistance of 195 ± 3.2 M Ω ($n = 35$). When the cells were dialyzed with the internal pipette solution containing Csfluoride, the resting membrane potential depolarized by ~ 40 mV.

NMDA-receptor-mediated response includes a late polysynaptic component in the presence of bicuculline

In the first set of experiments, we studied the effect of GABA_A-receptor blockade on pharmacologically isolated NMDA_R-mediated field potentials evoked by orthodromic stimulation of the Schaffer collaterals/commissural pathway (see Fig. 1A). These responses were recorded extracellularly in the stratum radiatum of the CA1 region, in low extracellular Mg²⁺ concentration (0.3 mM, to promote NMDA_R-mediated responses). As previously reported (Asztely et al. 1992; Gozlan et al. 1994), electrical stimulation evoked conventional monosynaptic field potentials (Fig. 2). When GABA_A-receptor-mediated inhibition was blocked by 10 μ M bicuculline, similar electrical stimuli evoked a more complex response composed of an early and a late component (Fig. 2). Interestingly, the late responses did not occur spontaneously. The late component had typical features of a polysynaptic network-driven event (Ben-Ari and Gho 1988; Johnston and Brown 1984; MacVicar and Dudek 1983; McLean et al. 1995; Wong and Traub 1983): its time-to-peak decreased with increasing stimulation intensities, whereas that of the early phase remained constant (Fig. 2) and it was blocked by high

Ca²⁺ medium (7 mM), which preferentially abolishes the polysynaptic response (Fig. 2) (Miles and Wong 1987; Wong and Traub 1983). In keeping with the facilitatory effect of Ca²⁺ on synaptic transmission (Dodge and Rahamimoff 1967), in the presence of the high Ca²⁺ ACSF, the initial slope of the monosynaptic field potential was significantly increased (by $66.2 \pm 22.8\%$, $P = 0.028$, $n = 6$).

To isolate the polysynaptic response, the monosynaptic field potential obtained in high Ca²⁺ conditions was subtracted from the total field potential recorded in normal Ca²⁺ concentration. As the monosynaptic field potential increased in high Ca²⁺ concentration, the stimulation intensity was decreased to normalize its amplitude (see METHODS) with that recorded in normal Ca²⁺ concentration. The resulting traces show the isolated polysynaptic component (Fig. 2). Its latency (ranging from 10 to 55 ms, $n = 5$) and its amplitude varied with stimulation intensity. In contrast, its time to peak (27.3 ± 3.0 ms, $n = 5$) and its half amplitude duration (60.7 ± 5 ms, $n = 5$) were relatively constant (Fig. 2). Hereafter, we will refer to this polysynaptic response as NMDA-polysynaptic response.

Thus after blockade of GABA_A receptors, a graded NMDA-polysynaptic response can be generated by electrical stimulation in the isolated CA1 region in the presence of 0.3 mM Mg²⁺.

Study of the local circuit driving the NMDA-polysynaptic response

In the second set of experiments, we studied whether recurrent collaterals are involved in the generation of the

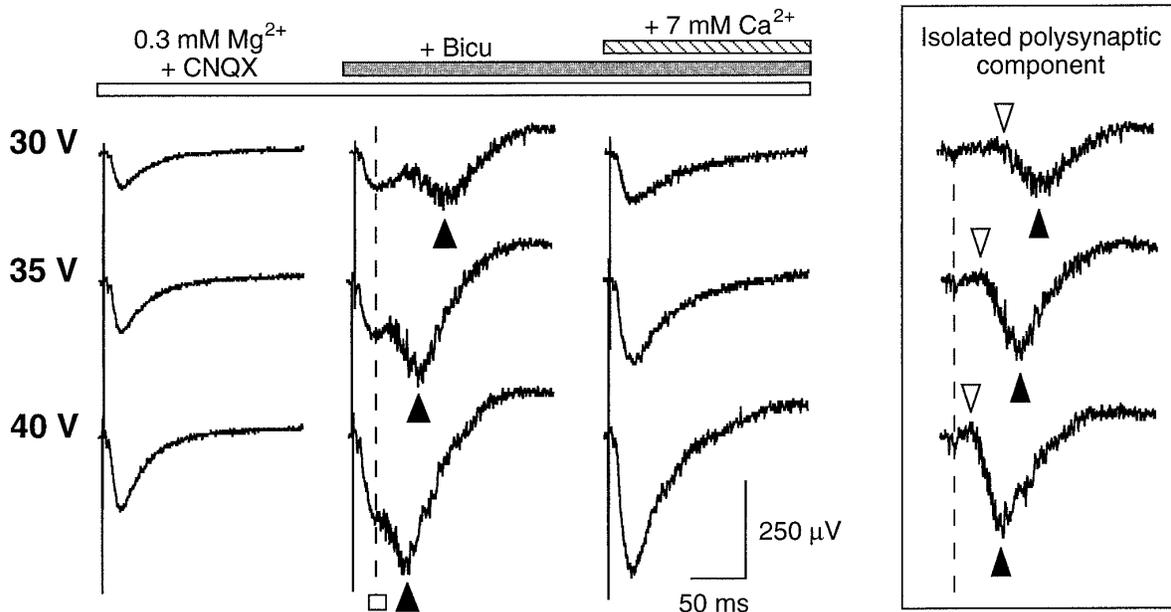


FIG. 2. *N*-methyl-D-aspartate-receptor (NMDA_R)-mediated polysynaptic responses evoked in the presence of bicuculline. NMDA_R-mediated field potentials evoked by orthodromic stimulations (30, 35, and 40 V) and recorded extracellularly in stratum radiatum of CA1 in low external concentrations of Mg²⁺ (0.3 mM) and in the presence of 10 μ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and glycine. In this and following figures, traces are average of 5 individual traces. Field potentials were recorded before (*left*), after application of 10 μ M bicuculline (*middle*), and after subsequent addition of 7 mM Ca²⁺ (*right*). Note that time to peak of late component (\blacktriangle) decreased with increasing stimulation intensities in contrast to early component (\square). *Insets*: show isolated polysynaptic component obtained by subtraction of normalized field potentials recorded in presence of bicuculline plus 7 mM Ca²⁺ from responses recorded only in presence of bicuculline (*middle*). Note that latency of isolated polysynaptic component (\triangle) decreased with increasing stimulation intensities in contrast to its time to peak, which varied only slightly (\blacktriangle).

local excitatory response. If so, the polysynaptic response should be evoked by antidromic stimulation (Miles and Wong 1986). The extracellular recording electrode was placed in stratum radiatum of the CA1 region, and the stimulating electrode in the alveus of the CA1 region (to antidromically activate the axons of CA1 pyramidal cells, see Fig. 1B). In these experiments, two additional knife cuts were performed in the slices (into the stratum oriens and the stratum radiatum, between the recording and the stimulating electrodes) to prevent orthodromic synaptic activation of CA1 pyramidal cells. Without bicuculline, electrical stimulation evoked only axonal volley (Fig. 3A). Because we cut the alveus and the oriens (see Fig. 1B), this volley might reflect the electrical activity along local recurrent collaterals and not the antidromic action potentials propagating along the CA1 pyramidal cell axons. In the presence of bicuculline (10 μ M), the same electrical stimulation generated a volley followed by a negative field potential (Fig. 3A). These responses were fully abolished by D-APV (50 μ M; not shown) and thus were generated by NMDA_R. The antidromically evoked slow field potentials were similar to the polysynaptic responses evoked by orthodromic stimulation: their time to peak and their half-amplitude duration were 23.7 ± 3.5 ms ($n = 5$) and 54.2 ± 5.7 ms ($n = 5$), respectively, and their maximal amplitude and

their latency (ranging from 4.3 to 20 ms, $n = 5$) varied with the intensity of stimulation (Fig. 3A). In addition, increasing the external concentration of Ca²⁺ (to 7 mM) strongly suppressed these field potentials, confirming that they were mediated by polysynaptic connections (Fig. 3A).

In a second approach to study the local circuit driving the NMDA- polysynaptic responses, we focally applied (by pressure injection) 30 μ M of TTX in the stratum oriens to inhibit the propagation of action potentials along the initial part of the pyramidal cell axons, thus preventing the activation of recurrent collaterals. As shown in Fig. 3B, a large part of the late synaptic component, evoked by the stimulation of the Schaffer collateral/commissural pathway, was abolished by TTX. In the presence of TTX, the surface area of the total synaptic responses was reduced by $40 \pm 2\%$ ($P = 0.0016$, $n = 5$). The effect of TTX was however not due to a reduction of the firing in the Schaffer collateral/commissural pathway or to a reduction of the monosynaptic responses, because TTX did not significantly change the amplitude of the afferent volley (by $-4.2 \pm 2.6\%$, $P = 0.094$, $n = 5$) or the slope of the early phase of the field potential (by $-5.5 \pm 2.7\%$, $P = 0.065$, $n = 5$; Fig. 3B).

These results provide evidence that recurrent collaterals are involved in the generation of the NMDA polysynaptic response.

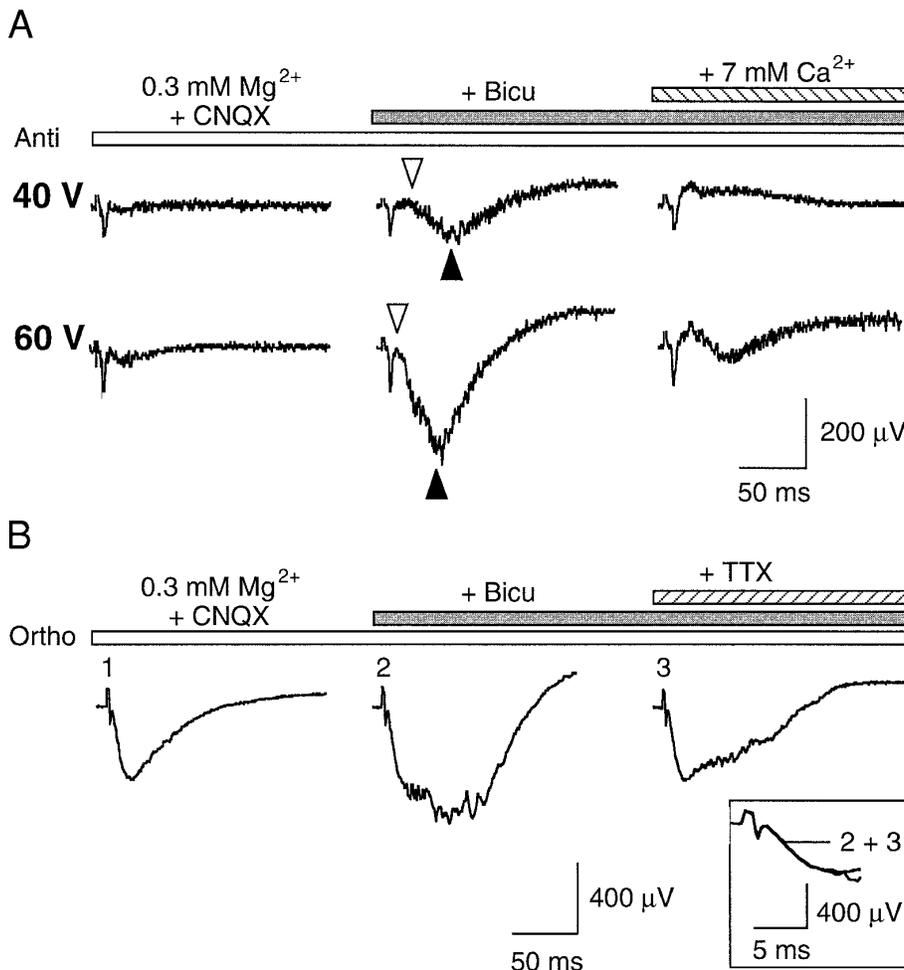


FIG. 3. Polysynaptic NMDA_R-mediated field potentials evoked by antidromic and orthodromic stimulation. A: NMDA_R-mediated field potentials evoked by antidromic stimulations (40 and 60 V) and recorded extracellularly in stratum radiatum of CA1 in low external concentrations of Mg²⁺ (0.3 mM) and in the presence of 10 μ M CNQX. Responses were recorded before (left), after application of 10 μ M bicuculline (middle), and after subsequent addition of 7 mM Ca²⁺ (right). Note appearance of field potentials in presence of bicuculline. Also note that latency of onset (∇) decreased with increasing stimulation intensities in contrast to time to peak (\blacktriangle). B: NMDA_R-mediated field potentials evoked by orthodromic stimulation and recorded extracellularly in stratum radiatum of CA1 in low external concentrations of Mg²⁺ (0.3 mM) and in the presence of 10 μ M CNQX. Synaptic responses were recorded before (1), after application of 10 μ M bicuculline (2), and after subsequent focal application of 30 μ M tetrodotoxin (TTX) in stratum oriens of CA1 (3). Insets: show superimposed traces 2 and 3 at a fast time base. Note that focal application of TTX in oriens strongly reduced late polysynaptic response without changing afferent volley and initial slope of the field potential.

Spatial distribution of the excitatory synapses driving polysynaptic responses

In an attempt to determine the spatial distribution of the excitatory synapses driving polysynaptic responses along the CA1 dendrites, a CSD analysis was performed. The NMDA-polysynaptic response was evoked by antidromic stimuli (Fig. 1B). The recording electrode was moved along a track perpendicular to the stratum pyramidale, from the lacunosum moleculare to the oriens (Fig. 4A). The pyramidal cell layer was taken as reference. Because the polysynaptic response may arise from the activation of a wide band of fibers, we used a sampling interval of 50 μm to be able to detect a discrete synaptic sink. First, a laminar profile analysis was performed: the peak amplitude of the field potential was plotted against the recording distance from the pyramidal cell layer. The mean profile showed that there was a relatively homogeneous distribution of negative and positive field potentials along the CA1 pyramidal cells ($n = 5$, Fig. 4C). Second, the CSD was calculated and the resulting traces were shown in the Fig. 4B. The mean surface area of CSD (calculated within 50 ms from the shock artifact) was plotted against the recording distance from the pyramidal cell layer

(Fig. 4D). This CSD analysis showed that synaptic sinks were distributed between 50 and 150 μm below the pyramidal cell layer, in the stratum radiatum, with a peak at 50 μm ($n = 5$). The source was sharply limited to the pyramidal cell layer ($n = 5$).

These observations provide electrophysiological evidence that the antidromically evoked NMDA-polysynaptic response is driven by synapses impinging onto the proximal portion of the apical dendrites of CA1 pyramidal cells. However, we cannot exclude that synapses impinging onto the basal dendrites (masked by cutting through the oriens) also generate polysynaptic responses.

Topographical features of the inhibitory synapses controlling the synchronous discharge

To examine the spatial distribution of the inhibitory synapses preventing the generation of the NMDA-polysynaptic response, bicuculline (300 μM) was applied focally by pressure along a track perpendicular to the stratum pyramidale. The diffusion of bicuculline also was tested by applying bicuculline outside (but close to) the CA1 region, in the

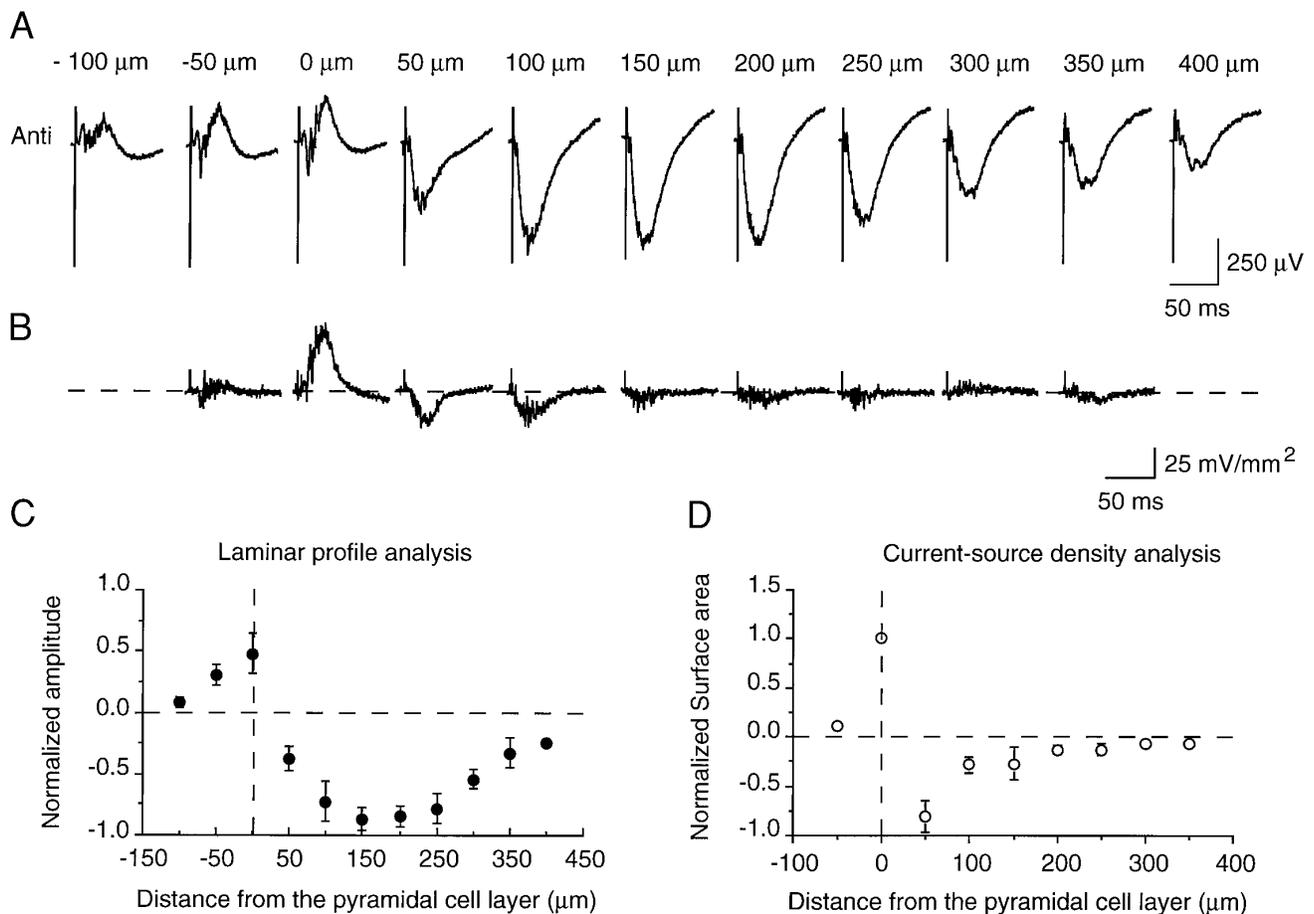


FIG. 4. Laminar profile and current-source analysis of antidromic-evoked NMDA_R-mediated field potentials. *A*: laminar profile of field potentials. Field potentials were evoked by antidromic stimulation and recorded extracellularly in low external Mg^{2+} (0.3 mM) in the presence of 10 μM bicuculline and 10 μM CNQX. Recording electrode was moved with a sampling interval of 50 μm along a track perpendicular to stratum pyramidale. *B*: traces obtained by current-source density (CSD) analysis. *C*: plot of normalized amplitude of synaptic responses ($n = 5$) vs. distance from pyramidal layer (maximal negative amplitude normalized to 1). *D*: plot of surface area (calculated within 50 ms from shock artifact) of CSD traces ($n = 5$) vs. distance from pyramidal layer (maximal negative amplitude normalized to 1).

lacunosum moleculare of the fascia dentata. The field potentials were evoked by orthodromic stimulation and recorded in the stratum radiatum. In six slices tested, polysynaptic responses were obtained by pressure application of bicuculline to stratum radiatum or lacunosum moleculare of the CA1 region, but not to stratum oriens (Fig. 5). As expected, there was also no polysynaptic responses when bicuculline was applied outside the CA1 region (in the lacunosum moleculare of the fascia dentata).

These results indicate that the inhibitory synapses that control the CA1 local excitatory circuit are distributed widely throughout stratum radiatum and lacunosum moleculare of the CA1 region. This observation is in keeping with electrophysiological data reporting GABAergic inhibition (Rovira et al. 1984) or GABA_A-receptor-mediated synaptic responses (Lacaille and Schwartzkroin 1988; Lambert et al. 1991; Pearce 1993) in the apical dendrite of CA1 pyramidal cells.

Current-voltage relation of the NMDA-polysynaptic excitatory postsynaptic current

We studied the *I-V* relation of the NMDA-polysynaptic response. In this and the following experiments, synaptic responses were evoked orthodromically and recorded in the CA1 pyramidal layer, in whole cell voltage-clamp mode. The amplitude of the early and late component were measured respectively at 3 and 120–150 ms after onset of the excitatory postsynaptic current (EPSC), and were plotted versus membrane holding potentials (Fig. 6, *A* and *B*). As shown in Fig. 6*B*, the *I-V* curves of mono- and polysynaptic currents were similar, both displaying the *I-V* relation typical of NMDA_R-medi-

ated currents. The mono- and polysynaptic currents reversed at 4.4 ± 1.7 mV ($n = 8$) and 5.0 ± 1.6 mV ($n = 8$), respectively, and their *I-V* relation displayed area of negative slope conductance at voltages more negative than -41.5 ± 2.1 mV ($n = 8$) and -43.2 ± 2.5 mV ($n = 8$), respectively.

NMDA-polysynaptic EPSC is blocked at physiological concentration of Mg²⁺

In previous experiments, the ACSF contained a low external concentration of Mg²⁺ (0.3 mM). The purpose of the following experiment was to establish whether the NMDA-polysynaptic response can be triggered in the presence of a normal external concentration of Mg²⁺ (1.3 mM). As shown in Fig. 6*C*, increasing the external concentration of Mg²⁺ from 0.3 to 1.3 mM abolished the late polysynaptic component. The remaining EPSC showed the typical features of the NMDA_R-mediated monosynaptic response (Khazipov et al. 1995; Perouansky and Yaari 1993). At -30 mV, the EPSC had a rise time (10–90%) of 9.4 ± 1.0 ms ($n = 9$), and its decay time was best fitted by a double-exponential function with both fast ($\tau_f = 53.9 \pm 5.5$, $n = 9$) and slow time constant ($\tau_s = 241 \pm 28.7$ ms, $n = 9$) (Fig. 6*D*). The *I-V* relation revealed that the equilibrium potential was 4.1 ± 1.1 mV ($n = 9$) and displayed a strong region of negative slope conductance at voltages more negative than -31 ± 1.1 mV ($n = 9$; Fig. 6*E*).

To test whether the block of the NMDA-polysynaptic response in 1.3 mM Mg²⁺ was due to a decrease in cell excitability (Berry and Pentreath 1976), the NMDA-polysynaptic response was recorded in 0.3 mM Mg²⁺ and 3 mM Ca²⁺

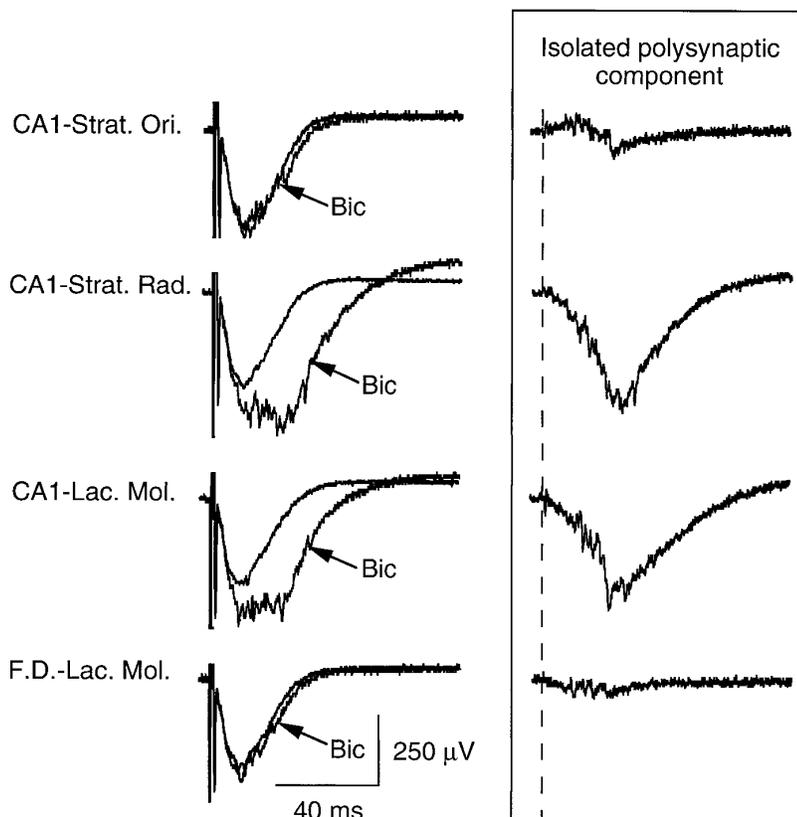


FIG. 5. Effects of focal application of bicuculline in various layers on NMDA_R-mediated field potentials. NMDA_R-mediated field potentials evoked by orthodromic stimulation and recorded extracellularly in stratum radiatum of CA1 in low external concentrations of Mg²⁺ (0.3 mM) and in the presence of 10 μM CNQX. *Left*: superimposed field potentials recorded in absence and presence of discrete pressure application of 300 μM bicuculline (Bic) in stratum oriens (Strat. Ori.), stratum radiatum (Strat. Rad.), lacunosum moleculare (Lac. Mol.) of CA1, and in lacunosum moleculare of fascia dentata (F.D.-Lac. Mol.). *Insets*: show isolated polysynaptic component obtained by subtraction of traces (*left*). Note that bicuculline evoked polysynaptic responses when applied in stratum radiatum or lacunosum moleculare of CA1.

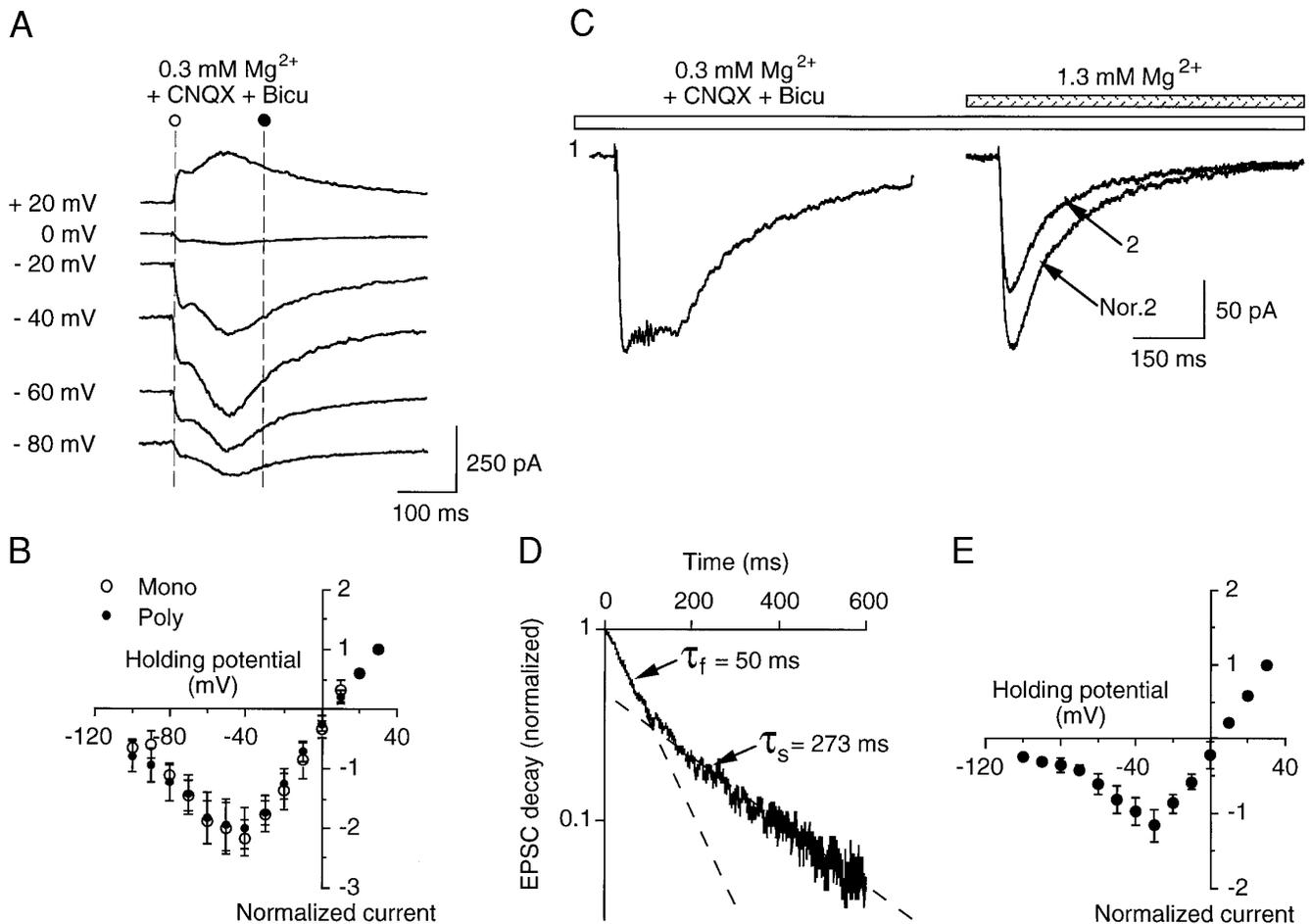


FIG. 6. NMDA_R-mediated polysynaptic responses recorded in whole cell configuration from CA1 pyramidal neurons. *A*: NMDA_R-mediated excitatory postsynaptic currents (EPSCs) recorded at different holding potentials in low external Mg²⁺ (0.3 mM) and in the presence of 10 μM of bicuculline and 10 μM CNQX. In this and following panels, EPSCs were evoked orthodromically and recorded in CA1 pyramidal cell layer in whole cell voltage-clamp mode. *B*: plot of monosynaptic (measured 4 ms after the onset of EPSC, ○) and polysynaptic (measured 180 ms after onset of EPSC, ●) component of synaptic responses (*n* = 8) vs. holding potentials (amplitude at V_h = +30 mV normalized to 1). Note that *I*-*V* curve of mono- and poly-EPSCs displayed *I*-*V* relation of NMDA_R-mediated current. *C*: EPSCs recorded at -30 mV before (1) and after increasing external concentration of Mg²⁺ from 0.3 to 1.3 mM (2). Because amplitude of EPSCs (2) was reduced in 1.3 mM Mg²⁺, stimulation intensity was increased to normalize EPSC amplitude (Nor. 2) to that of EPSC (1). *D*: semilogarithmic plot of Nor.2-EPSC decay (maximal negative amplitude normalized to 1) vs. time. Note that EPSC decay was fitted with a double exponential, with a fast (τ_f = 50 ms) and slow (τ_s = 273 ms) time constant. *E*: plot of amplitude of EPSC (recorded in 1.3 mM Mg²⁺, *n* = 9) vs. holding potentials (amplitude at V_h = +30 mV normalized to 1).

(instead of 2 mM) to maintain constant the external concentration of divalent ions. In this condition, the NMDA polysynaptic response was not reduced (*n* = 5; not shown). This result indicated that the disappearance of the polysynaptic component in 1.3 mM Mg²⁺ was not due to an overall change in cell excitability but was presumably due to a reduction of NMDA receptor-mediated responses in the local circuit.

Thus both a low extracellular concentrations of Mg²⁺ (0.3 mM) and a blockade of GABA_A-receptor-mediated inhibition are required to generate the pharmacologically isolated NMDA-polysynaptic response.

Blockade of GABA_A receptors induces mixed AMPA/NMDA-polysynaptic responses in normal concentrations of Mg²⁺

The preceding experiments were performed in the presence of an AMPA_R antagonist. We then investigated whether

a late polysynaptic current could be evoked in normal external concentrations of Mg²⁺ (1.3 mM) and in the absence of CNQX. As previously reported (Kandel et al. 1961), in the absence of bicuculline, electrical stimulation of the Schaffer/commissural pathway evoked synaptic responses consisting of an early EPSC followed by an inhibitory postsynaptic current (IPSC, not shown). In the presence of 10 μM bicuculline, the same electrical stimulation evoked an early and a late EPSC. This late EPSC, observed in all slices tested (*n* = 5), revealed features similar to the NMDA-polysynaptic response: its amplitude and its latency varied with the intensity of stimulation (see Fig. 7, *A* and *B*). The nature of the early and late component was studied by plotting their *I*-*V* relation. The *I*-*V* relation of the early component was measured at 3 ms (after onset of the EPSC) and that of the late phase at two latencies: at its peak (30 ms after onset of the EPSC) and at 100 ms after onset of the EPSC (Fig. 8*A*).

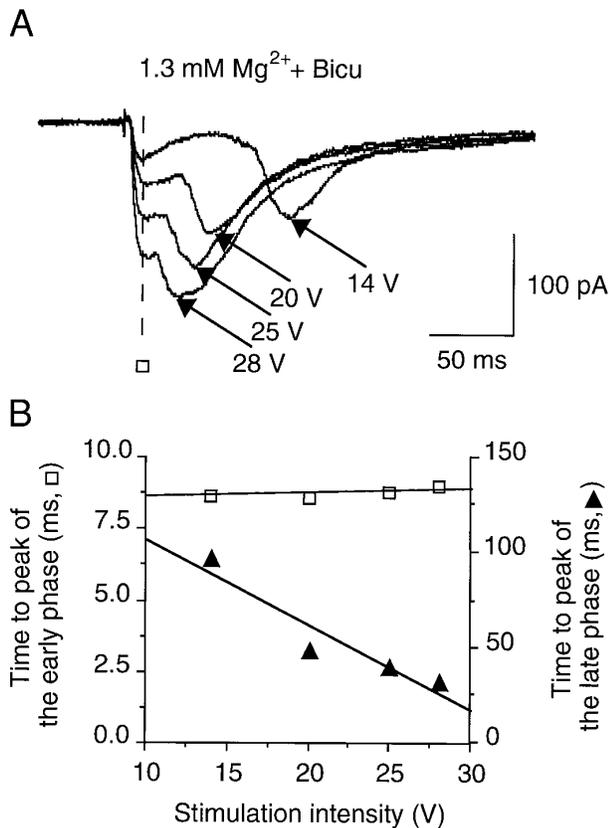


FIG. 7. In normal concentrations of Mg^{2+} and in the absence of CNQX, block of γ -aminobutyric acid-A ($GABA_A$) receptors induced a late synaptic response. *A*: EPSCs recorded at -60 mV and evoked by different stimulation intensities (14–28 V). In this and following panels, EPSCs were recorded in normal external concentrations of Mg^{2+} (1.3 mM) and in presence of $10 \mu M$ of bicuculline. Under those conditions, EPSCs included an early (\square) and a late component (arrow). *B*: plot of time to peak of early (\square) and late (\blacktriangle) components of EPSCs vs. stimulation intensity. Note that time to peak of late component decreased with increasing stimulation intensity in contrast to time to peak of early component.

The I - V curves of the early phase and the peak of the late component were similar, both displaying the typical linear I - V relation of AMPA_R-mediated currents (Fig. 8, *B* and *C*), and reversing close to 0 (at -0.18 ± 3.1 mV and 4.0 ± 2.6 mV, $n = 5$, respectively). The I - V curve of the synaptic response measured at 100 ms, also reversed close to 0 (at -1.25 ± 2.4 mV, $n = 5$), but was not linear and displayed an area of reduced slope conductance at voltages more negative than -32 ± 1.5 mV ($n = 5$), suggesting a large contribution of NMDA_R at this latency (Fig. 8*D*).

Thus in the absence of CNQX and in physiological concentrations of Mg^{2+} (1.3 mM), removal of $GABA_A$ inhibition reveals late synaptic components. The early phase and peak component of the late phase of the EPSC (measured at 30 ms) seem to be mediated mostly by AMPA_R. In contrast, the late phase of the late component (measured at 100 ms) seems to be mediated both by AMPA_R and NMDA_R.

NMDA receptors contribute to the generation of AMPA- and NMDA-receptor-mediated polysynaptic responses

In the presence of $10 \mu M$ bicuculline and 1.3 mM Mg^{2+} , addition of $50 \mu M$ D-APV did not significantly change the

early EPSC, as previously reported (Hestrin et al. 1990; Perkel and Nicoll 1993), confirming that it is mediated by AMPA_R (Fig. 8, *A* and *B*): its amplitude was changed by $+6.1 \pm 8.4\%$ ($P = 0.19$, $n = 5$) and $+3.5 \pm 19.6\%$ ($P = 0.18$, $n = 5$) at $V_h = -80$ and $+20$ mV, respectively. Interestingly, the late synaptic currents, measured at 30 ms and presumably AMPA_R-mediated (from its I - V curve, see above), were reduced strongly by $50 \mu M$ D-APV (Fig. 8, *A* and *C*): its amplitude was decreased by $51.5 \pm 9.8\%$ ($P = 0.007$, $n = 5$) and $54.6 \pm 5.3\%$ ($P = 0.0009$, $n = 5$) at $V_h = -80$ and $+20$ mV, respectively. As expected, the synaptic currents, measured at 100 ms, also were reduced strongly by D-APV: its amplitude was decreased by $70.45 \pm 1.5\%$ ($P = 0.0012$, $n = 5$) and by $80 \pm 2.7\%$ ($P = 0.0014$, $n = 5$) at $V_h = -80$ and $+20$ mV, respectively (Fig. 8, *A* and *D*).

Thus these results suggest that the synchronization of the local circuit involves NMDA_R. These observations also confirm that the late synaptic component (measured at 100 ms) is mediated partly by NMDA_R.

AMPA receptors mediate polysynaptic responses following removal of $GABA_A$ inhibition in normal concentration of Mg^{2+}

The AMPA_R-mediated late component of the EPSC, recorded in the presence of $50 \mu M$ D-APV, was polysynaptic because it was abolished in ACSF containing a high concentration of divalent cations (4 mM Ca^{2+} and 6 mM Mg^{2+}) (see Fig. 9*A*). The remaining EPSC showed the typical features of the AMPA_R-mediated monosynaptic response (Hestrin et al. 1990). First, it was blocked by CNQX (not shown). Second, its I - V relation was linear and reversed at 4.3 ± 1.6 mV ($n = 5$) (Fig. 9*C*). Third, at -80 mV its rise time (10–90%) and its decay time (best fitted with a simple-exponential function) was 3.15 ± 0.23 ms and 10.7 ± 0.96 ms ($n = 5$), respectively (Fig. 9*B*).

Thus in normal concentrations of Mg^{2+} and after removal of $GABA_A$ inhibition, activation of AMPA_R can generate a polysynaptic response.

DISCUSSION

Principal features of polysynaptic responses generated in the isolated CA1 region

The main finding of the present work is that, after removal of $GABA_A$ -mediated inhibition, electrical stimulation can evoke a glutamatergic polysynaptic response in the isolated CA1 area. This response is mediated by AMPA and/or NMDA receptors depending on the pharmacological conditions and the external concentration of Mg^{2+} used. First, we show that the late glutamatergic synaptic component, induced after removal of $GABA_A$ inhibition, possesses several features typical of the polysynaptic response. Its latency varied inversely with the stimulation intensity and it was abolished by a high external concentration of divalent cations (7 mM Ca^{2+} or 4 mM Ca^{2+} and 6 mM Mg^{2+}). Second, when the NMDA receptor-mediated response is pharmacologically isolated, the polysynaptic response can be evoked only in low external concentration of Mg^{2+} (0.3 mM). In contrast, if AMPA receptors are not blocked (by $10 \mu M$ CNQX),

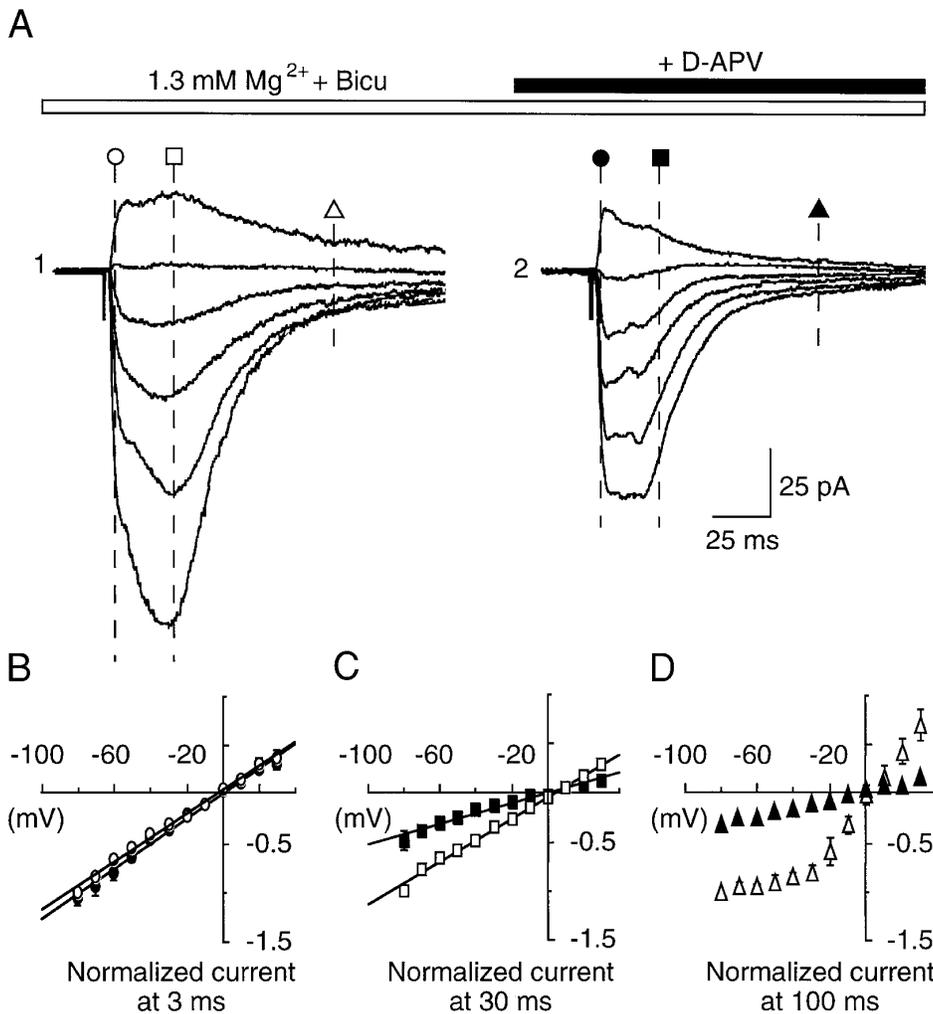


FIG. 8. NMDA_R contribute to generation of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-receptor (AMPA_R)-mediated polysynaptic response. *A*: superimposed traces of EPSCs recorded at different holding potentials (from -80 mV to $+20$ mV, by steps of 20 mV) before (1) and after application of D-2-amino-phosphonovalerate (D-APV, 2). *B–D*: plots of EPSC amplitude measured before (open symbol) and after application of D-APV (close symbol), at 3 ms (*B*, circle), at 30 ms (*C*, square), and at 100 ms after onset of the EPSC (*D*, triangle) ($n = 5$) vs. holding potentials (maximal negative amplitude normalized to 1). Note that *I–V* curves of the current measured at 3 and 30 ms displayed typical linear *I–V* relation of AMPA_R-mediated current. In contrast, *I–V* curve of current measured at 100 ms was not linear and displayed a region of reduced conductance at voltages more negative than -30 mV. Also note that APV strongly reduces polysynaptic component measured at 30 ms and presumably mediated by AMPA_R.

the polysynaptic response can be evoked in physiological concentration of Mg^{2+} (1.3 mM). Under these conditions, the polysynaptic response is mediated both by AMPA and NMDA receptors. We further provide two lines of evidence suggesting that the polysynaptic response is driven by local recurrent collaterals of CA1 pyramidal cells axons: blockade of Na^+ spikes in these collaterals (by using focal application of TTX in the stratum oriens) strongly reduced the polysynaptic component and antidromic stimulation of CA1 pyramidal cells could evoke the polysynaptic response.

Our findings are in agreement with the previous work of Thomson and Radpour showing that CA1 pyramidal cells are interconnected by local recurrent collaterals of their axons and these recurrent collaterals can generate AMPA- and NMDA-receptor-mediated synaptic responses (Deuchars and Thomson 1996; Thomson and Radpour 1991). We suggest that in the CA1 as in the CA3 region (Ben-Ari and Gho 1988; Christian and Dudek 1988; Miles and Wong 1987), removal of GABA_A inhibition will help to reveal latent excitatory recurrent synapses between CA1 pyramidal cells and generate the polysynaptic response. Interestingly, polysynaptic responses observed here are different than those previously reported for the CA3 region (Ben-Ari and Gho 1988; Johnston and Brown 1984; Miles and Wong 1987; Schwartzkroin and Prince 1978), because they are generated in a graded fashion and are not present spontane-

ously. The low level of interconnectivity between CA1 pyramidal cells (Deuchars and Thomson 1996; Thomson and Radpour 1991) and the low level of intrinsic spontaneous activity of these cells (Prince 1983) might explain the absence of spontaneous polysynaptic events. Because the polysynaptic response is graded, we suggest that it is generated by subsets of CA1 local circuit.

Histological studies, performed on Sprague-Dawley rats and guinea pigs, have shown that local collateral branches of CA1 pyramidal cell form a fine axonal plexus that synapses into the stratum oriens and the pyramidal cell layer (Amaral et al. 1991; Deuchars and Thomson 1996; Knowles and Schwartzkroin 1981). We therefore expected that synapses generating the polysynaptic response would impinge on the basal dendrites of CA1 pyramidal cells. In contrast, the CSD analysis of the NMDA-polysynaptic response indicated that, in Wistar rats, these responses are generated along the proximal part of the apical dendrites of CA1 pyramidal cells. This discrepancy may be due to differences between strains or species of animals: for example, mossy fibers form an infrapyramidal band in the CA3 area of Sprague-Dawley rats (West et al. 1981) but not in Wistar rats (Represa et al. 1987). Further histological studies of recurrent collaterals synapses between CA1 pyramidal cells of Wistar rat will be required to clarify this point.

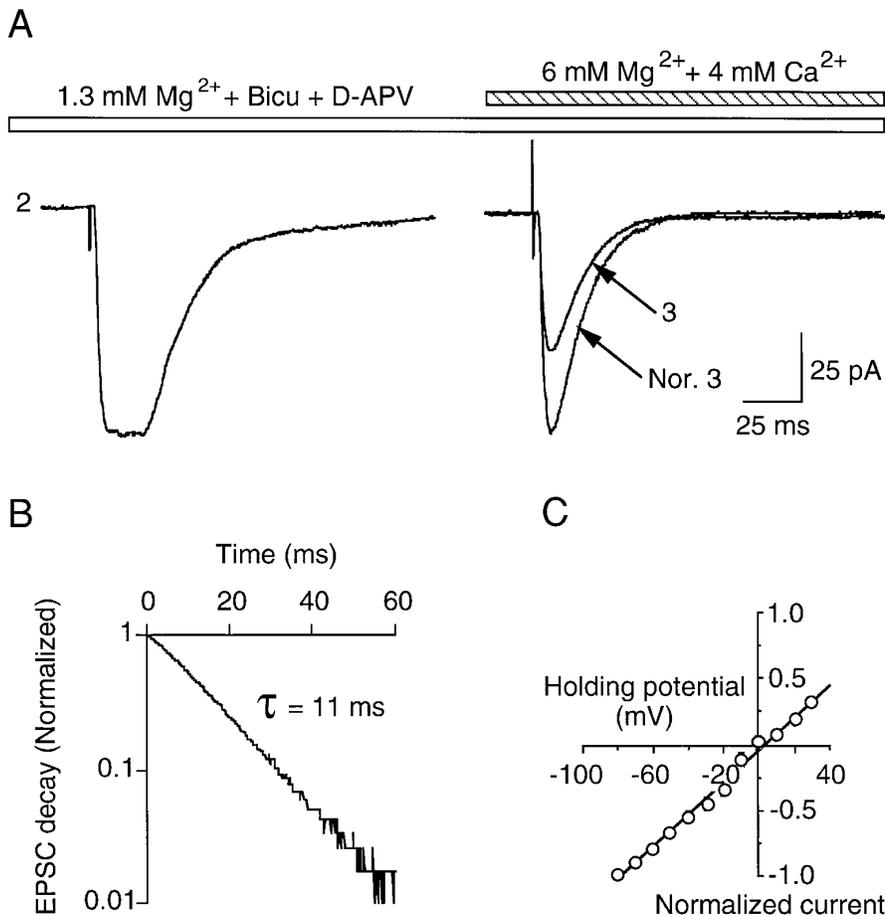


FIG. 9. Increasing external concentration of Mg^{2+} and Ca^{2+} , respectively, to 6 and 4 mM abolished polysynaptic component of AMPA_R-mediated EPSC. *A*: AMPA_R-mediated EPSC recorded at -80 mV in the presence of $10 \mu M$ bicuculline and $50 \mu M$ D-APV (trace 2 also shown in Fig. 8A). EPSCs were recorded before (2) and after increasing the external concentration of Mg^{2+} and Ca^{2+} to 6 and 4 mM, respectively (3). Because amplitude of EPSC (3) was reduced by 4 mM Ca^{2+} and 6 mM Mg^{2+} , stimulation intensity was increased to normalize EPSC amplitude (Nor. 3) to that of EPSC (2). *B*: semilogarithmic plot of Nor.3-EPSC decay (maximal negative amplitude normalized to 1) vs. time. Note that EPSC decay was fitted with a single exponential with a time constant $\tau = 11$ ms. *C*: plot of amplitude of EPSC (recorded in 6 mM Mg^{2+} and 4 mM Ca^{2+} , $n = 5$) vs. holding potentials (maximal negative amplitude normalized to 1).

Finally, the present study suggests that NMDA_R contribute to the generation of AMPA/NMDA_R-mediated polysynaptic responses. We found that the polysynaptic response likely mediated by AMPA_R (from the *I-V* curve, see RESULTS) are depressed strongly in the presence of a NMDA_R antagonist. A possible explanation is that NMDA_R may facilitate the synchronization of the network because the response mediated by these receptors have a higher probability of summation (due to their slower kinetics) in comparison with the fast AMPA_R-mediated response. At resting membrane potential, the NMDA_R-mediated current is reduced strongly by the voltage-dependent Mg^{2+} block (see Fig. 6E). We propose that, in normal concentrations of Mg^{2+} , the AMPA_R-mediated postsynaptic potential depolarize the cells sufficiently to activate NMDA_R. This hypothesis is supported by the fact that, without activation of AMPA_R, the polysynaptic response can only be evoked in low external concentrations of Mg^{2+} (a condition in which the NMDA current is increased in the negative range of potentials, see Fig. 6B).

Spatial distribution of inhibitory synapses controlling the local excitatory circuit

The topographical feature of inhibitory synapses controlling the CA1 local excitatory circuit also have been examined. We show that the polysynaptic response is evoked when inhibitory synapses are blocked along the apical dendrites of CA1 pyramidal cells. These results indicate that

inhibitory synapses that control the local excitatory circuit are distributed widely throughout stratum radiatum and lacunosum-moleculare region of the CA1 area. The present results are in keeping with previous reports showing that many types of interneurons innervate proximal and distal portions of apical dendrites of CA1 pyramidal cells (Buhl et al. 1994; Kawaguchi and Hama 1987; Lacaille and Schwartzkroin 1988; Lambert et al. 1991; Sik et al. 1995; Williams et al. 1994) and that the dendritic inhibition is involved strongly in the control of CA1 pyramidal cell excitability (Alger and Nicoll 1982; Masukawa and Prince 1984).

The importance of other types of interneurons (such as basket and axo-axonic cells, which innervate either the soma or the initial segment, respectively) (Buhl et al. 1994; Li et al. 1992; Sik et al. 1995; Thurbon et al. 1994) in the control of the local excitatory circuit cannot be excluded, because the bicuculline also may diffuse in the stratum pyramidale when the application was performed closed to this area.

Implication of polysynaptic responses in physiological and pathological activities of CA1 pyramidal cells

The present study provides electrophysiological evidence that, after removal of GABA_A inhibition, recurrent synapses between CA1 pyramidal cells form a local excitatory circuit that can drive synchronized discharges. The physiological role of this local excitatory circuit is not clear. However, it has been proposed that recurrent syn-

apses between CA1 pyramidal cells play an important role in hebbian processes (Hebb 1949), because they are involved in the induction of long-term potentiation (Radpour and Thomson 1991). Another possibility is that the CA1 local excitatory circuits participate in pathological processes. Thus glutamatergic graded bursts are observed in the CA1 region, both in an acute *in vitro* (Williamson and Wheal 1992) and chronic models of epilepsy (Turner and Wheal 1991). However, *in vivo* after epileptic seizures, the local excitatory circuits driving the synchronous discharges may be reinforced by a sprouting of local axon collaterals of CA1 pyramidal cells (Perez et al. 1996). It has been found recently that after an anoxic-aglycemic episode, a polysynaptic component is revealed (Crépel and Ben-Ari 1996; Tsintsadze et al. 1996). We suggest that, in pathological conditions such as epilepsy or ischemia, the CA1 local excitatory network may be unmasked and lead to a persistent and noxious hyperexcitability.

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