Activity-Dependent Induction of Multitransmitter Signaling Onto Pyramidal Cells and Interneurons of Hippocampal Area CA3

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INTRODUCTION

The normally glutamatergic granule cells of the dentate gyrus (DG) monosynaptically excite pyramidal cells and interneurons in hippocampal area CA3 through their mossy fibers (MF). The interneurons are the main targets of the MF (Acsády et al. 1998); therefore after being excited, they inhibit pyramidal cells and other interneurons (Miles et al. 1996; Urban and Barrionuevo 1998). Thus excitatory and inhibitory synaptic responses in CA3 to MF stimulation disappear when glutamatergic transmission is blocked, indicating that GABAergic responses are disynaptically mediated (Buzsáki 1984; Crawford and Connor 1973; Dichter and Spencer 1969; Yamamoto 1972).

However, we recently demonstrated that after seizures (Gutiérrez 2000; Gutiérrez and Heinemann 2001) or after synaptic or direct kindling-like stimulation of the DG in vitro without inducing epileptiform activity (Gutiérrez 2002), MF activation evokes monosynaptic GABAergic potentials in pyramidal cells. This and immunohistochemical (Ramírez and Gutiérrez 2001; Schwarzer and Sperk 1995; Sloviter et al. 1996) and neurochemical evidence (Gómez-Lira et al. 2002; Taupin et al. 1994a,b) suggest that granule cells can synthesize and release GABA in an activity-dependent manner. Furthermore, we also demonstrated that the granule cells and their MF contain and express the vesicular GABA transporter (VGAT) mRNA in an activity-dependent manner (Lamas et al. 2001).

It is a general principle that all the terminals of a given neuron release the same neurotransmitter(s). However, because the MF are functionally compartmentalized (Maccarelli et al. 1998; Tóth et al. 2000), it is reasonable to consider the possibility that release of glutamate and GABA from MF could also be segregated according to the type of target onto which they impinge. Therefore to support the hypothesis that MF release glutamate and GABA, it was important to test whether the simultaneous glutamatergic and GABAergic transmission is expressed onto both MF targets in CA3: the pyramidal cells and the interneurons.

Interestingly, in a previous study (Gutiérrez and Heinemann 2001), we described that after the blockade of the aforementioned MF-evoked GABAergic response a putative cholinergic response could be uncovered. However, cholinergic transmission to area CA3 has been shown to come exclusively from the associational/commissural fibers (A/C), and its modulation in the MF projection is exerted through indirect synaptic interactions, whereby inhibitory interneurons are excited and presynaptically modulate MF glutamate release (Vogt and Regehr 2001). In view of this, we decided to characterize the seizure-induced DG-evoked cholinergic transmission onto CA3 and compare it to that evoked by the stimulation of the stratum radiatum, where the A/C pathway is known to pass (Vogt and

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A

CA3 interneuron

CA3 pyramidal cell

Mossy fibers

Glu

GABA

control

NBQX + APV

100ms

5 mV

20 mV

100ms

2 nA

100ms

50ms

Glu

GABA

control

NBQX + APV

100ms

5 mV

10mV

100ms

50ms

B

PYRAMIDAL CELLS

INTERNEURONS

10 mV

20ms

10 mV

50 ms

10 mV

20ms

10 mV

50 ms

10 mV

50 ms

C

SL

CA3

SP

SO

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Regenhorn and Regehr 2001). This would also clarify how these responses interact in CA3 neurons.

Here we demonstrate that after a generalized convulsive seizure, DG activation evokes monosynaptic GABAergic and M1-cholinergic responses both in pyramidal cells and interneurons of CA3, through the activation of different pathways, each with a distinctive pharmacological profile that permits their selective presynaptic modulation. Their emergence depends on protein synthesis.

**Methods**

**Electrophysiological recordings and analysis**

For our electrophysiological experiments, we used control and pentylenetetrazol (PTZ)-injected Wistar rats (230–250 g). The intraperitoneal injection of PTZ (Sigma; 70 mg/kg) invariably induced a generalized convulsive clonic-tonic seizure that lasted 80–120 s, after which myoclonias and wet-dog shakes were apparent. The rats were decapitated under deep ether anesthesia. All experimental procedures were approved by the Committee on Ethical Animal Research of our Institution and of the Ministry of Health. Brains were rapidly dissected and combined entorhinal cortex-hippocampus slices (400 μm) were cut with a vibro slicer (Campden Instruments, England) submerged in oxygenated artificial cerebrospinal fluid (ACSF) at 4°C. The PTZ-treated group was decapitated 1 h after the injection. For some experiments, slices from control and PTZ-treated rats were incubated in artificial cerebrospinal fluid (ACSF) in the presence of cycloheximide (60 μM) or anisomycin (100 μM) (Jones et al. 1992; Otani et al. 1992) for ≥2 h before they were transferred to the recording chamber. All slices, including those preincubated with the protein synthesis blockers, were transferred to a moisturized air-liquid interface recording chamber where they were constantly perfused with oxygenated normal ACSF at 35°C for 1 h before the recordings started. The ACSF contained (in mM) 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, 26 NaHCO₃, and 10 glucose; pH 7.35. The drugs used were diluted at the desired concentration in the ACSF, namely: the nMDA type glutamate receptor antagonist, (2R,3S)-2-amino-5-phosphonoveric acid (APV; 30 μM; Tocris), and the non-nMDA type glutamate receptor antagonist 6-nitro-7-sulfamoylbenzo(f)quinolaxine-2,3-dione (NBQX; 10 μM; Tocris); the γ-aminobutyric receptor antagonist bicuculline methiodide (20 μM; Sigma); the GABA₆ receptor antagonist CGP55845A (1 μM; a gift from Novartis); the M₁-cholinergic antagonists: pirenzepine (10 μM; RBI), atropine (10 μM; Sigma), and muscarinic toxin (MT7; 100 nM; Peptide Institute); the M2-cholinergic agonist oxotremorine (1 μM; Sigma); the group II mGluR agonist (2S,3R)-2′-carboxycyclopentylglycinic acid (DCG-IV; 1 μM; Tocris); the group III mGluR agonist (±)-2-amino-4-phosphonobutyric acid (t-A4P; 10 μM; Tocris); and the protein synthesis inhibitors, cycloheximide (60 μM; Sigma) and anisomycin (100 μM; Sigma).

An hour after incubation in normal ACSF, intracellular recordings were done with an AxoClamp 2B amplifier (Axon Instruments) and borosilicate microelectrodes (70–90 MΩ; Brown-Flaming Puller) filled with 2M potassium acetate and 2% biocytin (Sigma). Electrophysiologically identified pyramidal cells were recorded from regions CA3a and -b, and interneurons were all recorded from the marginal zone between the stratum lucidum and s. pyramidale of region CA3b (Fig. 1). Anatomical identification of the recorded cells was later carried out by the intracellular injection of biocytin. The signals were acquired and analyzed off-line with the program pClamp8 (Axon Instruments). The duration of the action potential, and the duration and amplitude of the afterhyperpolarization were measured as previously described (Scharfman 1995). The onset latency of the synaptic responses was measured from the beginning of the stimulation artifact to the first inflection point of the first derivative of the synaptic potential with the cursor measure function of Clampfit. Membrane input resistance, rise time, and time to peak of the synaptic responses were measured with the built-in functions of Clampfit. The results are expressed as means ± SE.

Electrical stimulation was provided with bipolar glass-insulated platinum wire (50 μm) electrodes over the molecular layer of the DG and over the s. radiatum of the CA3 region (Vogt and Regenhorn 2001) (Fig. 1A). Pulses of 0.1 ms were delivered at a current intensity that evoked 75% of the excitatory postsynaptic potential (EPSP) amplitude needed to reach threshold for evoking action potentials. In the CA3 region, a glass pipette (1–2 MΩ) filled with ACSF was positioned 200–300 μm apart from the recorded cell to stimulate local interneurons with monopolar pulses of 0.1 ms.

**Immunohistological experiments**

MF INTRACELLULAR LABELING. Slices to which a crystal of dextran, fluorescein (MW 3000; Molecular Probes) was inserted in the granular cell layer of the DG with a patch pipette were perfused with ACSF for 5 h and immersed in phosphate buffer (0.1 M; pH 7.4) with paraformaldehyde (4%) for 12 h and then transferred to a buffer with sucrose (30%) for 24 h. They were frozen and cut at 14 μm in a cryostate (Leica, CM 1850) and recovered in gelatin-coated slices. Some slices were counterstained with Evans blue (Sigma). These were covered with Vectashield (Vector) and observed and photographed under an epifluorescence microscope with appropriate filters for FITC and Texas Red (Nikon Optiphot-2).

For detection of cholacin-acetyltransferase (ChAT) immunoreactivity, four slices from 2 PTZ-treated rats and from two control rats were processed after MF dextran, fluorescein staining. The slices were fixed in phosphate buffer (0.1 M; pH 7.4) with paraformaldehyde (2%) and picric acid (0.18%) for 12 h and transferred to a buffer with sucrose (30%) for 24 h. The slices were frozen, cut, and mounted as described in the preceding text. Sections were preincubated with BSA (5%) for 2 h and incubated with goat polyclonal anti-ChAT (human) antibody (1:200; Chemicon) in PBS, Triton X-100 (0.3%) and BSA (5%) overnight in a humid atmosphere at room temperature. For control purposes, some sections were simultaneously processed in the absence of the primary antibody. After rinsing, they were incubated with biotinylated anti-goat IgG (1:100; Vector) 1 h, followed of avidine-Texas Red (1,200, Vector) 1 h. Finally sections were coverslipped with Vectashield fluorescein mounting medium (Vector) and examined with an epifluorescence microscope. Selected sections were analyzed with a scanning confocal microscope (MRC 1024 Bio-Rad) equipped with an argon/krypton laser. Images of the preparations stained with dextran, fluorescein were acquired with an excitation wavelength of 488 nm and ChAT Texas-red fluorescence with 568...
nm. Acquisitions for each wavelength were done separately over the same fields and the combined renditions were digitally processed with the computer program Confocal Assistant (Tood Clark Brelje). The red signal corresponds to ChAT Texas red fluorescence and the green signal to dextran, fluorescein fluorescence. Colocalization of both signals would render a yellow labeling.

RESULTS

Typical responses of pyramidal cells to depolarizing pulses showed a burst of two or three action potentials followed by a hyperpolarization. On the other hand, interneurons recorded in the marginal zone between the stratum lucidum and stratum pyramidale of region CA3b were characterized by a sustained firing of action potentials during the depolarizing pulse, with little or no accommodation (Fig. 1, A and B). Cells that did not meet these criteria were not included in our study. The duration and shape of the action potential were distinctive for each cell type. The characteristics of the action potential, the resting membrane potential (rmp) and input resistance of both types of cells in control and PTZ-treated preparations are summarized in Table 1.

Synaptic responses of pyramidal cells and interneurons to MF activation in control preparations

DG stimulation evoked in pyramidal cells and interneurons EPSP/inhibitory postsynaptic potential (IPSP) sequences, which were completely blocked by the perfusion of non-N-methyl-D-aspartate (NMDA) and NMDA receptors’ antagonists (NBQX and APV; Fig. 1, A and B). The values of the amplitude, onset latency, time to peak, and rise time of the monosynaptic control EPSP in pyramidal cells and interneurons are summarized in Table 1.

MF activation provokes GABAergic synaptic responses in pyramidal cells and interneurons after seizures

On DG stimulation, the pyramidal cells of the PTZ group responded with EPSP/IPSP sequences (Figs. 2, A1, and C1, and 3A1). In interneurons, however, only an EPSP was evident (Figs. 2, A2 and C2, 3A2). The values of the amplitude, onset latency, time to peak, and rise time of the monosynaptic EPSP in pyramidal cells and interneurons are summarized in Table 1. As compared with the control preparations, the amplitude, rise time, and time to peak of the EPSP evoked in both types of cells were statistically different in the PTZ group (Student’s t-test; P < 0.01; the stimulation intensity was normalized as described in METHODS).

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<th>Table 1. Statistical comparison PTZ vs. control</th>
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<td>DG-IPSP amplitude, mV with ACh-antag.</td>
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Student’s t-test; values are means ± SE; n values are in parentheses. AHP, afterhyperpolarization; DG, dentate gyrus; EPSP and IPSP, excitatory and inhibitory postsynaptic potential; A/C, associational/commissural. * P < 0.05; ** P < 0.01.
rise time of the MF IPSP in pyramidal cells and interneurons are summarized in Table 1. The reversal potential of the IPSP obtained in pyramidal cells (−67 ± 0.7 mV; n = 25) and in interneurons (−69.4 ± 0.6 mV; n = 10; Fig. 2C, I and 2) was consistent with GABAergic responses, as also evidenced by its blockade with biccuculline (Fig. 3A, I and 2).

DG stimulation evokes cholinergic responses in CA3 after seizures

In contrast to control slices, in PTZ-treated preparations the stimulation of the DG in the presence of glutamate receptors’ antagonists and biccuculline provoked a slow depolarizing potential (EPSPm), both in pyramidal cells (Fig. 3A1) and interneurons (Fig. 3A2). The mean amplitude, rise time, duration, and onset latency for this EPSPm both in pyramidal cells and interneurons are summarized in Table 1. On higher stimulation intensity, this EPSPm was enhanced but did not evoke action potentials in neither of the CA3 targets (not shown).

We then compared the EPSPm evoked by stimulation of the DG to that evoked by stimulation of the A/C fibers (Vogt and Regehr 2001). The characteristics of the A/C-evoked EPSPm are depicted in Table 1. Figure 3B shows the experimental arrangement and the responses to DG and A/C stimulation. Perfusion of glutamate receptors’ antagonists block all synaptic responses to DG stimulation in control animals, but A/C stimulation evoked an EPSPm that could be pharmacologically isolated by perfusion of glutamatergic and GABAergic synaptic potentials in pyramidal cells and interneurons in response to DG stimulation. Superimposed synaptic responses of pyramidal cells (A1) and interneurons (A2) evoked in normal artificial cerebrospinal fluid (ACSF) at different stimulus intensities in control and pentylenetetrazol (PTZ)-treated preparations (postseizure). On stimulation intensity increments, the EPSP of the pyramidal cells (A1) of the control group increased, whereas the IPSP barely changed. On the contrary, in the PTZ-treated group the EPSP presented a more pronounced increment as compared with the EPSP. A2: the interneurons showed the opposite behavior, whereby in the control group, the IPSP augmented as the stimulus intensity was increased (rmp: −65 mV) and in the PTZ-treated group, stimulus intensity increments evoked a more pronounced EPSP and no IPSP could be detected (rmp: −65 mV).

B: superimposed fast synaptic responses in normal ACSF and during perfusion of glutamate receptors’ antagonists of a pyramidal cell (B1; rmp: −66 mV) and of an interneuron (B2; rmp: −67 mV) in a PTZ-treated preparation. C: the synaptic responses evoked during the DC current pulses in normal ACSF behaved as expected for glutamatergic and GABAergic signals, however, during the perfusion of glutamate receptors’ antagonists, an IPSP with characteristics of a GABAergic signal could be isolated, and reversed at a potential consistent with GABA transmission in both MF targets. No difference in input membrane resistance was observed in PTZ-treated preparations as compared with controls. Traces in A and B are averages of 6–10 synaptic responses.
DCG-IV, and of the mGluR-III agonist, L-AP4 on the MF-evoked responses. We tested the effect of both agonists in a group of cells and found that the inhibitory effect of DCG-IV on the MF IPSP was milder (26%) than the effect of L-AP4, which depressed the MF IPSP by 84% \((n = 11; \text{Fig } 4, D \text{ and } E)\), coinciding with previously reported data (Gutiérrez 2000, 2002; Walker et al. 2001). This effect is selective for the MF-evoked IPSP because the inhibitory potentials evoked by direct stimulation of intrinsic interneurons of area CA3 were not affected \((n = 6; \text{Fig } 4D)\). Moreover, we found that L-AP4 perfused during the maximal effect of DCG-IV further inhibited the MF IPSP \((\text{Fig } 4F)\). Thereafter, the effect of L-AP4 was

**Fig. 3.** DG stimulation simultaneously evokes a glutamatergic, a GABAergic, and a cholinergic potential both in pyramidal cells and interneurons of PTZ-treated rats. A: in normal ACSF, DG stimulation evokes EPSP/IPSP sequences in pyramidal cells (A1; rmp: $-65 \text{ mV}$) and interneurons (A2; rmp: $-67 \text{ mV}$). During perfusion with 6-nitro-7-sulfamoylbenzof(2,3-dione (NBQX) + (DL)-2-amino-5-phosphonovaleric acid (APV), a fast IPSP was isolated, which could be blocked by bicuculline. Its blockade revealed a slow EPSP that could be blocked with M1-cholinergic antagonists. The onset latencies of the EPSP, IPSP, and EPSPm evoked in each type of neuron are depicted in the corresponding bar graphs. B1: schematic representation of the hippocampus showing the sites of stimulation used to activate the associational/commissural (A/C) and the MF pathways. B2: synaptic responses of control CA3 pyramidal cells to DG and A/C stimulation (rmp: $-66 \text{ mV}$). Perfusion of glutamate receptors' antagonists block all synaptic responses evoked by DG stimulation but isolate GABAergic potentials evoked by direct stimulation of interneurons within stratum radiatum. Perfusion of bicuculline and CGP55845A isolates an EPSPm, which was blocked by pirenzepine. C: in PTZ-treated preparations, both, DG and A/C stimulation evoke IPSPs during perfusion of glutamate receptors' antagonists. The perfusion of GABA\(_\text{A}\) and GABA\(_\text{B}\) antagonists isolates an EPSPm, which is completely and reversibly blocked by the highly selective M1-ACh\(_\text{R}\) toxin, MT7 (rmp: $-65 \text{ mV}$). D: the input resistance of the cell augmented during the EPSPm, as revealed by passing hyperpolarizing current pulses (rmp: $-66 \text{ mV}$). This effect was blocked by pirenzepine.
tested in the GABAergic synaptic responses in the first and last cells recorded from each slice (we usually recorded 3–4 cells per slice). L-AP4 inhibited the MF-IPSP to the same extent in 44 of 44 cells tested.

Because L-AP4 has the ability to depress the MF-evoked IPSP, therefore in the presence of glutamate receptors’ antagonists, it should depress the IPSP and would uncover the underlying EPSPm, provided that the latter is not sensitive to glutamatergic and B-agonists, it should depress the IPSP and would uncover the underlying EPSPm.

L-AP4 strongly inhibits the IPSP recorded in interneurons (93). Also, the GABA B-dependent IPSPs evoked by direct interneuronal stimulation (INT) are modulated by 10 synaptic responses.

To study the presynaptic modulation of the different cholinergic responses of pyramidal cells evoked by DG and A/C stimulation, EPSPm were evoked successively in the same pyramidal cells by alternating the site of stimulation (as depicted in Fig. 3B) in the presence of glutamate and GABAergic antagonists (n = 93). Also, the GABA B-dependent IPSP was blocked with CGP55845A (n = 12). Under these conditions, we found that DCG-IV virtually blocked the GABAergic responses evoked by DG but not the A/C-evoked cholinergic responses, which remained unaltered (n = 5; Fig. 5, A and B). The responses to a paired pulse potentiation protocol showed that the effects are consistent with a presynaptic action of DCG-IV (Fig. 5B). Thus the activation of mGluR-II, but not of mGluR-III, inhibits the DG- but not the A/C-driven EPSPm. Due to this, one can think that MF could also be mediating the cholinergic potential. However, because, unlike extrinsic cholinergic fibers, MF do not express M2 receptors, we tested the effect of the M2 agonist, oxotremorine, on the EPSPm evoked by stimulation of both pathways. Oxotremorine completely and reversibly depressed both EPSPm (n = 5; Fig. 5, C and D), showing that the cholinergic transmission from the DG to CA3 is not of MF origin and, contrary to the A/C cholinergic responses, is modulated by mGluR-II. For comparison purposes, we assessed the effect of oxotremorine on the fast glutamatergic DG-evoked EPSP of control preparations and confirmed its lack of effect (n = 5; Fig. 5D).

Emergence of nonglutamatergic signaling from the DG to CA3 after seizures depends on protein synthesis

Because the induction of the MF IPSP in vitro depends on protein synthesis (Gutiérrez 2002), we tested the effect of cycloheximide and anisomycin, protein synthesis blockers, on the seizure-induced nonglutamatergic signaling from the DG to CA3. After preincubation of the slices from seizing-rats in the protein synthesis blockers (see Methods), synaptic potentials were evoked by DG and direct interneuron stimulation in pyramidal cells (9 for each inhibitor) and interneurons (n = 3; anisomycin). The perfusion of glutamate receptors’ antagonists blocked all synaptic responses evoked by DG but not by direct stimulation of local interneurons in all the pyramidal cells and interneurons tested (Fig. 6). For comparison purposes, we carried out the same experiments in slices from control non-treated rats and from slices prepared from PTZ-treated rats 3 h after the onset of the seizure. In the former, EPSP/IPSP sequences could normally be evoked by DG stimulation. The perfusion of glutamate receptors’ antagonists blocked all synaptic potentials evoked by DG stimulation, whereas an IPSP was evoked by direct interneuron stimulation, as previously shown in control nontreated slices (n = 5 for each inhibitor). On the other hand, in slices prepared 3 h after the seizure and then incubated in the blockers, nonglutamatergic transmission from DG to CA3 could still be evoked (not shown).
ChAT-positive fibers pass through the DG and along MF in CA3

After anatomically identifying the MF with topical application of dextran, fluorescein into the DG (Fig. 7, A and B), we carried out immunofluorescence staining to ChAT (Fig. 7, C–E). Control experiments, in which slices were processed in the absence of primary antibody, did not present immunoreactivity (not shown). We were able to discard the colocalization of ChAT and dextran, fluorescein in the granule cells or their MF both in control and PTZ-treated preparations. The perisomatic region of pyramidal cells and granule cells was densely immunoreactive to ChAT, where ChAT positive terminals were evident (Fig. 7C, 1 and 2). Also, fine fibers could be localized in stratum lucidum (Fig. 7C2). In addition, we found few cholinergic interneurons in the molecular layer of the DG (Fig. 7D1), whose orientation and terminals were restricted to the

FIG. 6. The emergence of non glutamatergic signaling evoked in pyramidal cells and interneurons by DG stimulation after seizures depends on protein synthesis. Contrary to PTZ-treated preparations incubated in normal ACSF (A), preincubation of the slices in cycloheximide prevents the appearance of nonglutamatergic DG-evoked potentials in the presence of glutamate receptors’ antagonists (B). Anisomycin, another potent protein synthesis blocker, also prevents the appearance of nonglutamatergic DG-evoked potentials in the presence of glutamate receptors’ antagonists both in pyramidal cells (C) and in interneurons (D) recorded from the same slice. By contrast, direct stimulation of interneurons within CA3 (INT) still elicited IPSPs in the same cells. Traces are an average of 6–10 synaptic responses.
DG. Fibers positive to ChAT could be traced passing through the molecular (Fig. 7D1) and granular cell layers (Fig. 7D2). We found no apparent differences in ChAT staining between the control (Fig. 7, C1 and D1) and PTZ (Fig. 7, C2 and D2) groups.

**DISCUSSION**

Our results show that seizures induce simultaneous glutamatergic and GABAergic transmission from the MF to both their targets in CA3: the pyramidal cells and interneurons. Together with these signals, an M1-AchR-mediated cholinergic response develops that affects both targets, where the synaptic signals interact. The emergence of these DG-evoked GABAergic and cholinergic signals requires protein synthesis, and each is subjected to selective presynaptic modulation. This and our immunohistological and confocal microscopy experiments demonstrate that the cholinergic signal has an origin different from the mossy fibers. Figure 8 summarizes our conclusions.

**Seizures induce simultaneous glutamatergic and GABAergic transmission from MF to its targets in area CA3**

Supporting the hypothesis that MF are able to release GABA, we show that the different targets of MF in area CA3 respond with simultaneous glutamatergic and GABAergic potentials. In control preparations, glutamate receptors’ antagonists block all synaptic responses to DG stimulation both in pyramidal cells and interneurons, indicating that GABAergic potentials are disynaptically mediated. However, slices of the PTZ-treated group presented MF-evoked GABAergic responses in a condition in which all excitatory components are blocked, discarding polysynaptic contamination (Sik et al. 1994; Weisskopf and Nicoll 1995). However, there is evidence of the presence of GABAergic inputs to CA3, other than intrinsic interneurons, and of interneurons within area CA3c that can reach the hilar region (Sik et al. 1997). Although activation of these inputs cannot be entirely ruled out, the possibility that the GABAergic responses seen in our experiments are of interneuronal origin seems unlikely for three main reasons. 1) Stimulation of the hilar (Gutiérrez and Heinemann 2001) and of the granule and molecular layers of the DG (Gutiérrez 2000, 2002; Gutiérrez and Heinemann 2001) of slices of healthy nontreated animals does not produce GABAergic responses on pyramidal cells and interneurons of areas CA3a,b, however, after seizures or LTP-like stimulation, these responses appear. 2) The projections of the interneurons with CA3 targets are really CA3 interneurons, restricted to CA3c. This type of neuron, described by Sik et al. (1997) is in CA3 s. oriens and only few dendrites project to the hilus proper. 3) In slices from nontreated animals, after completely

FIG. 7. ChAT immunofluorescence disclose a cholinergic plexus in the DG and CA3 but the MF are ChAT free. A: panoramic view of a hippocampal slice showing the MF stained with dextran, fluorescein. B: the regions depicted in the next panels. C: in this preparation of a control rat, MF and their thorny spines (in green) surrounded by a plexus of ChAT-positive terminals and fibers (in red) in s. lucidum of CA3. No colocalization of the dextran, fluorescein and ChAT-positive signals was observed. D: some (ChAT-positive) cholinergic fibers could be followed and appeared to run parallel to the MF pathway. D1: scattered cholinergic (ChAT-positive) interneurons were consistently observed in the s. molecular of the DG, whose processes remained within the limits of this region or reached the stratum granulosum. D2: some (ChAT-positive) cholinergic fibers could be seen passing throughout the s. granulare. C1 and D1 correspond to control rats, whereas C2 and D2 to PTZ-treated rats. Calibration bars: B1, 100 μm; B2, 20 μm; C and D, 50 μm. SL, s. lucidum; SM, s. molecular; SG, s. granulare; SP, s. piramidale, H, hilus.
blocking synaptic responses by the perfusion of glutamate receptors’ antagonists, LTP-like stimulation of the DG induces the emergence of GABAergic responses. These are sensitive to mGlur activation and to protein synthesis blockade (Gutiérrez 2002), whereas GABAergic responses evoked by direct interneuron stimulation are not. This evidence strongly supports the notion of an activity-dependent mechanism and discards the possibility of stimulating interneurons projecting from the molecular layer of the DG to as far as CA3a,b.

Accordingly, the control EPSP and the pharmacologically isolated MF-evoked IPSP had the same latency, as previously reported (Gutiérrez 2000, 2002; Gutiérrez and Heinemann 2001; Walker et al. 2001). The activation of mGlur reduces neurotransmitter output from MF (Manzoni et al. 1995; Min et al. 1998; Salin et al. 1996) but has no effects on GABAergic transmission from interneurons in the DG (Tong et al. 1996) and in CA3 (this work; Walker et al. 2001). Thus that the latency of the IPSP isolated by the perfusion of glutamate receptors’ antagonists is not affected in the presence of mGlur agonists, or of baclofen, a GABA<sub>A</sub> agonist known to inhibit neurotransmitter release from MF and interneurons (Brown and Johnston 1983; Gutiérrez 2002), and even when perfusing them in a low-Cu<sup>2+</sup> medium (Gutiérrez 2000) provide strong evidence that our recordings represent monosynaptic IPSPs of MF origin. Further support to the hypothesis that MF can release GABA is provided from works showing that GAD (Ramírez and Gutiérrez 2001; Schwarz and Sperk 1995; Sloviter et al. 1996), GABA (Gómez-Lira et al. 2002), and VGAT mRNA (Lamas et al. 2001) are expressed in the granule cells and their MF in an activity-dependent fashion. This is further confirmed by our experiments showing that protein synthesis is required to trigger the MF GABA-releasing process (see also Gutiérrez 2002). However, the cellular mechanisms by which the blockade of protein synthesis disrupts the expression of nonglutamatergic signaling cannot be inferred from our present results.

Walker et al. (2001) have found this MF inhibitory transmission to be normally present in young guinea pig slices. Although marked differences are known to exist in regard to MF physiology in the two species, we have recently determined (R. Gutiérrez, H. Romo-Parra, J. Maqueda, M. Ramírez, M. A. Morales and M. Lamas, unpublished results) that MF GABAergic signaling is strongly linked of mGluR-III to the glutamate releasing machinery, which has been considered that MF glutamatergic neurotransmission can be recognized by its sensitivity to mGluR-III activation. It was considered that MF glutamatergic neurotransmission in the rat is sensitive to the mGluR-II agonist, DCG-IV, but not to the mGluR-III agonist, L-AP4. The contrary is true for MF neurotransmission in the guinea pig (Bradley et al. 1996; Lanthorn et al. 1984; Shigemoto et al. 1997; but see Ohishi et al. 1993, 1995). That MF GABAergic signaling is strongly inhibited by the activation of the mGluR-III permits to suggest a link of mGluR-III to the GABA-releasing machinery and of mGluR-II to the glutamate releasing machinery, which has been shown to produce a downregulation of the exocytotic
The granule cells and MF of the rat DG express groups II/III mGluR mRNA (Ohishi et al. 1993, 1995) and mGluR2,4,7 (Bradley et al. 1996; Lie et al. 2000; Shigemoto et al. 1997). Electron microscopy has revealed immunolabeling for the mGluR-III predominantly in presynaptic active zones of asymmetrical and symmetrical synapses, whereas mGluR-II immunolabeling was found in preterminal rather than terminal portions of axons (Shigemoto et al. 1996,1997). Our data suggest a presynaptic segregation of mGluR receptors according to the class of neurotransmitter to be released. Postsynaptically, GABA_A receptors cluster apposed to glutamatergic terminals in cultured pyramidal hippocampal cells suggesting that there is an element common to GABA and glutamate synapses (Rao et al. 2000).

Seizures induce mGluR-sensitive cholinergic transmission from the DG to pyramidal cells and interneurons in area CA3

Cholinergic responses of pyramidal cells and interneurons of the hippocampus usually appear on high-frequency or -intensity stimulation of their cholinergic afferents (Brunner and Misgeld 1994; Cole and Nicoll 1984; Morton and Davies 1997; Morton et al. 2001; Müller and Misgeld 1986), whereas single-pulse stimulation over the DG in the presence of glutamate receptors’ antagonists does not elicit synaptic responses in control preparations. However, after seizures, single-pulse stimulation over DG evokes M1-AChR-dependent responses, suggesting that these fibers are probably silent in normal conditions, and they become transiently active after seizures (Gutiérrez and Heinemann 2001). We now establish that this is a process dependent on protein synthesis.

It has been described that the cholinergic input from the septum exerts its modulation in the MF pathway through the activation of interneurons, which in turn affect pyramidal cells (Martin and Alger 1999; Williams and Johnston 1990; Vogt and Regehr 2001). Our data show that M1-cholinergic signaling of DG origin directly activates both pyramidal cells and interneurons within CA3. This, contrary to the A/C evoked-cholinergic responses, is sensitive to activation of mGluR-II, whereas M2-AChR activation depresses ACh release from both pathways. It has been described that activation of mGluR-II depresses MF but not A/C synapses (Manzoni et al. 1995) and muscarine directly inhibits the A/C synapse without directly affecting the MF synapse (Vogt and Regehr 2001), which does not have M2 receptors (Hájos et al. 1998), as we also confirm.

The cholinergic activation of different subsets of neurons and/or interneurons and, as we show, the activation of different cholinergic pathways with differential presynaptic modulation, regulate the overall synaptic integration in the hippocampus (Hasselmo and Feblau 2001). Thus in PTZ-treated preparations, the DG-cholinergic signal postsynaptically modulates the convergent glutamatergic and GABAergic neurotransmission. In this way, this adds to other modulatory mechanisms in a pathway where spill over is a means by which collateral multi-target modulation is exerted together with pre- and postsynaptic interactions in a parallel fashion (Hájos et al. 1998; Min et al. 1998; Rusakov et al. 1999; Semyanov and Kullmann 2000; Vogt and Regehr 2001).

Anatomical findings

Our immunohistological data discard the presence of ChAT in the MF and disclose a pathway that probably runs in parallel to this projection. As previously shown (Frotscher and Léránth 1985), we found ChAT-positive fibers to form a network around granule cells in the DG and pyramidal cells and interneurons in CA3. The terminals observed in the CA3 region are likely to be collaterals of those innervating the DG. In accordance with previous reports (Blaker et al. 1988; Frotscher et al. 2000), we observe the presence of few cholinergic interneurons, whose arborizations terminate locally within the s. molecular of the DG. Additionally, Frotscher et al. (2000) showed that neither GAD_{65} nor GAD_{67} mRNA colocalizes with ChAT in cholinergic interneurons.

Functional implications

Our present data showing that MF glutamatergic and GABAergic neurotransmission can be observed both in pyramidal cells and interneurons and that MF GABAergic signaling has a distinctive presynaptic modulation suggest that distinct MF fiber collaterals are involved in MF GABAergic neurotransmission (Gutiérrez 2002). Its emergence has net inhibitory effects on pyramidal cells because the activation of MF at high frequencies provokes the more pronounced IPSPs in pyramidal cells of PTZ-treated rats to summate, hyperpolarizing the cells (Gutiérrez and Heinemann 2001). Although here we prove that MF GABAergic transmission is also expressed onto interneurons, which are the main targets of the MF (Acsády et al. 1998), we show that MF glutamatergic excitation surpasses the inhibitory transmission. Thus the interneurons are more readily excited than pyramidal cells, which continue to receive a strong inhibitory control from the interneurons within CA3, besides the MF GABAergic signal.

Beyond the attractive hypothesis of glutamate/GABA corelease from the MF, other important functional consequences arise. Our model (Fig. 8) proposes that selective activation of mGluR-II would inhibit excitatory transmission (glutamatergic from the MF, and cholinergic from the associated parallel fibers) but not MF inhibitory transmission, which in turn, can still exert presynaptic GABA\textsubscript{A}-R activation and postsynaptically act on pyramidal cells and interneurons. Contrariwise, activation of mGluR-III would selectively inhibit inhibitory transmission without consequences in excitatory transmission, which could still further activate presynaptic mGluR. Finally, short- and long-term changes in the cholinergic system due to epileptic and stress-related phenomena are known to exist (Ferencz et al. 2001; Friedman et al. 1996; Kaufer et al. 1998; Lupica and Berman 1988; Mingo et al. 1997, 1998; Serra et al. 1997), whether these are related phenomena needs to be disclosed.

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