
Activity-dependent expression of simultaneous glutamatergic and GABAergic neurotransmission from the mossy fibers in vitro.

INTRODUCTION

The granule cells of the dentate gyrus (DG) monosynaptically excite pyramidal neurons of area CA3 and local inhibitory interneurons, which inhibit CA3 pyramidal cells (Dichter and Spencer 1969; Miles and Wong 1987). Thus the blockade of glutamatergic transmission, by glutamate receptor antagonists (GluRAs) to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate and N-methyl-d-aspartate (NMDA) receptors, abolishes monosynaptic excitatory responses and polysynaptic inhibitory responses of pyramidal cells to stimulation of mossy fibers (MF). In addition, GluRAs also abolish excitatory responses induced by stimulation of associational commissural inputs (Weisskopf and Nicoll 1995).

The granule cells of the DG are known to be glutamatergic; however, glutamic acid decarboxylase (GAD), the limiting enzyme for the synthesis of GABA, or its mRNA is normally present in these (Sloviter et al. 1996) and other excitatory neurons (Cao et al. 1996). Yet, GABAergic transmission from MF is not normally observed with microelectrode recordings. Interestingly, granule cells transiently express mRNA for GAD after the occurrence of limbic seizures, which also show immunostaining for GABA (Lehmann et al. 1996; Sandler and Smith 1991; Schwarzer and Sperk 1995; Sloviter et al. 1996), and we recently reported that after seizures, simultaneous glutamatergic and GABAergic transmission in the DG-CA3 projection transiently appears (Gutiérrez 2000; Gutiérrez and Heinemann 2001). We have hypothesized that this change may serve to limit the risk of seizure generation.

The coexistence of GABA and glutamate in MF (Sandler and Smith 1991) and the evidence showing that long-term continuous stimulation of the perforant path (PP) is known to cause seizures and up-regulation of GAD synthesis in granule cells (Sloviter et al. 1996) suggested de novo synthesis of GABA. Thus the hypothesis that granule cells are potentially able to release GABA, besides glutamate, for fast neurotransmission is suggested. This hypothesis is also supported by recent evidence showing monosynaptic GABAergic responses of pyramidal cells to MF stimulation in guinea pig hippocampal slices (Walker et al. 2001). That GABAergic responses in hippocampal area CA3 of the rat are detected after seizures, but not in control conditions, suggests that this amino acid is released from the MF in an activity-dependent fashion. To
explore this, I decided to test whether simultaneous glutamatergic and GABAergic transmission could be induced in vitro, in the absence of epileptic activity, and whether it depended on protein synthesis. Therefore I tested whether synaptic or direct kindling activation of the DG could induce this phenomenon in experiments in which the same slice/cell was its own control and determined the spacial specificity of this phenomenon. Finally, I analyzed the expression of GAD in the DG of the same preparations in which the simultaneous glutamatergic and GABAergic transmission was induced.

METHODS

Adult Wistar rats (250 g) were decapitated under deep ether anesthesia, and combined entorhinal cortex-hippocampus slices (400 μm) were obtained and maintained in vitro at 35 ± 0.5°C. They were constantly perfused with oxygenated artificial cerebrospinal fluid (ACSF) containing (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 in the ACSF, namely the NMDA receptor antagonist (D,L)-2-amino-5-phosphonovaleric acid (APV; 30 μM; Tocris); the non-NMDA receptor antagonist 6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione (NBQX; 10 μM; Tocris); the GABAβ receptor antagonist bicuculline methide (20 μM; Sigma); the group II metabotropic GluR agonist (2S,2’R,3’R)-2-(2’,3’-dicarboxycyclopropyl)glycine (DCG-IV; 1 and 10 μM; Tocris); the group III metabotropic GluR agonist 1-(+)-2-amino-4-phosphonobutyric acid (1-AP4; 10 μM; Tocris); the GABAα receptor agonist baclophen (3 μM; Sigma); and the protein inhibitor, cycloheximide (60 μM; Sigma). A bipolar (tip separation 25 μm), glass-insulated platinum wire (50 μm) electrode (approximate resistance 0.6 MΩ) was placed over the PP or the granule cell layer of the DG for stimulation with pulses of 0.1 ms. Intracellular activity of pyramidal cells in CA3 was recorded with glass microelectrodes (50–80 MΩ) filled with potassium acetate (2 M) and an AxoClamp 2B amplifier. For evoking synaptic potentials, single-pulse electrical stimulation of the granule cell layer of the DG was delivered at an intensity that evoked an excitatory postsynaptic potential (EPSP) with 60–70% of the amplitude required to make the cell fire action potentials.

The kindling stimulation protocol consisted of three trains of 0.1-ms pulses at 100 Hz, with a duration of 1 s, and an intertrain interval of 1 min delivered every 15 min without varying the stimulus intensity. Between the kindling stimulation trials, synaptic responses were evoked with single pulses at 0.1 Hz over the granular cell layer of the DG. The signal was acquired and analyzed off-line with the program pClamp6 (Axon Instruments). For the experiments in which protein synthesis was inhibited, the slices were incubated in cycloheximide (60 μM) (Jones et al. 1992; Otani et al. 1992) 15 min before initiation of the kindling stimulation protocol and for as long as it lasted (3 h). Intracellular recordings began ≥30 min after its withdrawal and could last several hours. The kindling stimulus intensity used for these experiments was previously determined and set at a value needed to obtain 50% of the maximum amplitude of the DG population response to test pulses.

For detection of GAD1 immunoreactivity, a PP-stimulated slice and a sister nonstimulated slice, both taken from the same recording chamber, were processed simultaneously after completion of the electrophysiological experiment. In all, three stimulated and three nonstimulated slices were processed. The slices were fixed in phosphate buffer 0.1 M at pH 7.4 with paraformaldehyde at 4% for 48 h and transferred to a buffer with sucrose (30%) for 24 h. The slices were frozen and cut at 30 μm in a microtome and collected in PBS (0.9% NaCl). The slices were incubated free-floating in PBS with 0.3% H2O2 to block endogenous peroxidase activity. After rinsing for three periods of 10 min in PBS, they were incubated in 10% horse serum (GIBCO), 1% gelatin (Sigma), and 0.2% Tween-20 (Sigma), to avoid unspecified GAD staining. Control slices were then incubated in the presence of GAD1 antibody (Chemicon) at 1:1,000 and stimulated slices at 1:1,500 in PBS and horse serum (5%; 4°C) during 36 h with agitation. After four 10-min washing periods, they were incubated in the presence of an anti-rabbit biotinylated IgG (1:800; Amersham Pharmacia Biotech). The slices were then rinsed four times and processed with the ABC Kit (Vectastain, Vector Laboratories) for 1 h at room temperature. After four 10-min washing periods, the slices were developed with diaminobenzidine (Sigma: 10 mg/25 ml PBS 10 mM) for 10 min and contrasted with nickel sulfate (30%).

RESULTS

In control conditions, test pulse stimulation of the DG evoked in pyramidal cells a fast monosynaptic EPSP followed by polysynaptic fast (GABAα-dependent) and slow (GABAβ-dependent) inhibitory postsynaptic potentials (IPSPs; Fig. 1, A and B). The onset latency of the evoked EPSP, measured from the beginning of the stimulus artifact to the beginning of the rising phase of the EPSPs, was 4.8 ± 1.0 ms (mean ± SE, n = 50). Simultaneous perfusion of the AMPA/kainate receptor antagonist NBQX and the NMDA receptor antagonist APV completely blocked both the EPSP and IPSPs in the control cells (n = 50; Fig. 1B). Synaptic responses remained blocked in the presence of GluRAs during depolarization of the cell (Fig. 1C) or after increasing the stimulus intensity to a value that previously evoked action potentials.

![FIG. 1. A: basic synaptic circuitry of the dentate gyrus (DG)-to-CA3 projection. DG stimulation induces a glutamatergic monosynaptic excitatory postsynaptic potential (EPSP) onto pyramidal cells and interneurons, which in turn inhibit pyramidal cells. B: EPSP/IPSP sequence evoked in a control pyramidal cell by DG stimulation. The perfusion of glutamate receptor antagonists (GluRAs) blocks the monosynaptic EPSP and the inhibitory postsynaptic potentials (IPSPs), demonstrating their polysynaptic origin. C: during perfusion of GluRAs, hyperpolarized or depolarized membrane potentials did not reveal synaptic responses to DG stimulation in control preparations. Traces in B are an average of 6 responses. The dots signal the stimulus artifact.](Image 346x156 to 522x436)
In vitro kindling experiments were then carried out as follows. Kindling stimulation was delivered 12 times to the PP under perfusion of normal ACSF, and extracellular activity in the DG and CA3 was monitored. The stimulus intensity was fixed at a value that evoked 50% of the maximum extracellular population synaptic response in CA3. This kindling protocol induced potentiation of the population synaptic responses both in DG and CA3 by around 250% in the DG and by around 50% in CA3, but no epileptiform activity was ever evoked \((n = 14; \text{Fig. 2A})\). After completion of the kindling protocol, intracellular activity was recorded from pyramidal cells, and synaptic responses were then evoked by single-pulse DG stimulation. The control EPSP, obtained prior to GluRAs perfusion had an amplitude of \(4.3 \pm 0.7 \text{ mV} (\text{mean} \pm \text{SE})\) and a mean rise time \((10\text{–}90\%)\) of \(2.7 \pm 0.4 \text{ ms}\) \((n = 20)\). On perfusion of GluRAs, the excitatory component was blocked uncovering a fast IPSP in 33 of 36 pyramidal cells tested \((\text{Fig. 2B})\). The IPSP mean amplitude, as evoked with the same current intensity used to evoke the control synaptic responses was \(2.4 \pm 0.2 \text{ mV}\), and its mean rise time was of \(7.7 \pm 0.6 \text{ ms}\) \((n = 33)\). Figure 2B shows intracellular recordings after completion of the kindling process, prior to \((\text{control})\) and after perfusion of GluRAs \((\text{nbqx} + \text{apv})\). The EPSP was blocked by perfusion of GluRAs, and a fast IPSP with a similar latency was isolated \((\text{Fig. 2B})\). The DG-induced IPSP evoked while current clamping the cell at different membrane potentials revealed a reversal potential consistent with a GABAergic IPSP \((\text{Fig. 2C} \text{; reversal potential} \; -68 \pm 0.5 \text{ mV}; \text{resting membrane potential} \; -64 \pm 2 \text{ mV}; n = 26)\). This was confirmed by its blockade with the GABA\(_A\) antagonist bicuculline \((\text{Fig. 2D})\).

We next decided to test whether the fast IPSP induced by synaptically activating the DG, via the perforant path, could also be obtained by the direct activation of the DG in the absence of glutamatergic transmission. Besides answering this question, the experimental approach used would permit the recording of the same cell before and after the emergence of the inhibitory potentials and to determine the time course of its appearance. Thus after recording control synaptic responses in a given pyramidal cell, we blocked them with GluRAs and applied the kindling stimulation protocol directly over the DG \((\text{Fig. 3A})\). Between the kindling trials \((15\text{-min interval})\), we delivered test single pulses over the same site at 0.1 Hz. After 16.6 \pm 0.9 kindling trials, a fast IPSP appeared on test pulses in 20 of 24 cells. The IPSPs could be completely blocked by bicuculline \((\text{Fig. 3A})\). At the time of its appearance, the IPSP amplitude was of \(1.5 \pm 0.2 \text{ mV} (n = 20)\) and further kindling stimuli resulted in potentiation of the IPSP \((n = 3; \text{Fig. 3B})\). In the other four cells, it was not apparent after 20 high-frequency stimulation trials. This stimulation protocol did not produce changes either in the input resistance \((33.8 \pm 1.7 \text{ MO} \Omega; \text{mean} \pm \text{SE})\) or in the resting membrane potential \((-64 \pm 2 \text{ mV}; n = 18)\).

Although 12 PP stimulation trials were effective for the induction of this phenomenon, it is possible that fewer kindling trials are needed to trigger the mechanism by which the GABAergic responses appear, making the following trials unnecessary for the completion of the readily triggered underlying intracellular processes. Therefore to test this, just four kindling trials were provided \((1 \text{h})\), and recordings were made 3 h after the onset of the kindling protocol in three slices. Once the control synaptic responses were obtained, GluRAs were perfused and a complete blockade of the EPSP/IPSP sequences was observed in 21 of 21 pyramidal cells tested \((\text{Fig. 4B})\), without observing any other remaining synaptic component.

Since baclophen hyperpolarizes interneurons and, additionally, it presynaptically acts on MF and interneurons to inhibit neurotransmitter release, experiments under perfusion of GluRAs plus baclophen were conducted to confirm that the evoked responses in CA3 are not the result of interneuron activation \((\text{Brown and Johnston 1983})\). In four of four cells, baclophen \((3 \text{ mM})\) perfused together with GluRAs hyperpolarized the pyramidal cells by around 10 mV. Under this condition, an IPSP could still be evoked by DG stimulation, but its amplitude, measured at the same membrane potential that the cell had before baclophen perfusion by current-clamping the cell, was reduced by \(92 \pm 1\%\). The onset latency, however,
FIG. 3. Kindling of the DG during glutamatergic transmission blockade induces inhibitory responses in CA3 to test pulse DG stimulation. A: superimposed responses of a pyramidal cell to single pulse DG stimulation during GluRAs perfusion before and after 18 trials of DG-kindling and its blockade by perfusion of bicuculline (RMP = −64 mV). B: superimposed responses to DG stimulation after 18 and 20 trials showing that stimulation beyond the appearance of the IPSP induces further potentiation of the responses. C: in the presence of GluRAs, baclophen depressed the DG-evoked IPSP without affecting its latency. Under this condition, polysynaptic contamination is unlikely. D: a paired-pulse protocol provoked facilitation of the 2nd IPSP. Perfusion of (2S,2R,3S)-2-(2R,3R-dicarboxycyclopropyl)glycine (DCG-IV) inhibited the 1st response, evidencing presynaptic inhibition of MF responses and favoring paired-pulse facilitation. E: plot showing the inhibitory effect and time course of the mGluR agonists L-2-amino-4-phosphonobutyric acid (L-AP4; group III; n = 7) and DCG-IV (group II; n = 8) on the DG-evoked IPSP. Notice a stronger depression by L-AP4 (mean ± SE). F: superimposed traces of the IPSP evoked at different moments before and during the perfusion of L-AP4, as indicated by numbers in the plot (RMP = −64 mV) and after the perfusion of bicuculline (trace 4). Test pulses were delivered at 0.1 Hz over the DG. Traces in A, B, C, and E are an average of 10 responses; in D, of 20 responses. Dots signal the stimulus artifact. The arrows signal the afferent volley.

FIG. 4. The activity-dependent induction of DG-evoked inhibitory potentials requires protein synthesis. A, left panel: the kindling stimulation protocol. Right panel: the development of the field potential responses of the DG to PP high-frequency stimulation along the kindling process. B: stimulation of the slices with an “incomplete” kindling protocol (only 4 trials) fails to induce inhibitory synaptic responses in CA3 on DG stimulation. C: the complete kindling stimulation protocol, which normally induces DG-evoked IPSPs in CA3 pyramidal cells, is not effective when applied in the presence of the protein synthesis inhibitor, cycloheximide. Each example in B and C shows the same cell before and after GluRAs perfusion, which blocks all synaptic potentials. Traces in A and B are averages of 10 responses.
was not altered (Fig. 3C). The onset latencies of the DG-evoked EPSP before GluRAs perfusion and the DG-evoked IPSP after GluRAs perfusion were 4.8 ± 1.0 ms (n = 35) and 5.2 ± 0.9 ms (n = 24; mean ± SD), respectively (Fig. 2B). The latencies of the EPSP and IPSP are not statistically different (Student’s t-test).

It is known that activation of metabotropic glutamate receptors (mGluR) reduces the output of glutamate from MF (Maccaferri et al. 1998; Manzoni et al. 1995). This has been used as an identification test for synaptic responses of MF origin (Manzoni et al. 1995; Min et al. 1998; Tong et al. 1996). According to this, the specificity of the MF signal was tested by a paired-pulse protocol, and the group II mGluR agonist DCG-IV was perfused. DCG-IV perfusion (for 10 min) produced a maximal inhibition of the IPSP of 30 ± 0.9% (mean ± SE; n = 8) in all the tested cells and produced potentiation of the second response, evidencing presynaptic inhibition of MF (Fig. 3D). Given the lack of complete inhibition of the IPSP by DCG-IV and previous data showing inhibition of the seizure-induced DG-evoked IPSP by L-AP4 (Gutiérrez 2000), the latter was also tested in seven cells. The maximal inhibition produced by L-AP4 (drug pulse of 10 min) was of 73 ± 0.3% (mean ± SE; n = 7) in all the cells tested. Figure 3E shows the time course and relative efficacy of both mGluR agonists to inhibit the DG-evoked IPSP. In contrast, as expected, GABAergic potentials elicited by direct interneuron stimulation within CA3 were not affected by perfusion of mGluR agonists (not shown).

To corroborate the spatial selectivity of the kindled pathway to induce inhibitory responses, we conducted a series of experiments in which cells were recorded and synaptic responses obtained with the following stimulation protocol. Once the pyramidal cell was impaled, two different sites of the granular cell layer of the DG (200–300 μm apart from each other) were stimulated to evoke independently EPSP/IPSP sequences. Usually, both electrodes were placed on the upper blade of the DG, close to the point where both blades converge. Figure 5, A and B, shows a schematic representation of these experiments, where, for the purpose of clearly illustrating two MF inputs, the sites of stimulation are depicted rather separate from each other. Synaptic responses were then blocked by perfusion of GluAs (Fig. 5A, NBQX + APV). Thereafter, kindling stimulation was delivered to one DG site, but not to the other, and test pulses were delivered to both sites to monitor the appearance of synaptic potentials. Interestingly, inhibitory potentials could be evoked by test pulse stimulation of the kindled site after 17 ± 1 kindling trials, but test pulse stimulation of the alternative site remained ineffective (n = 4; Fig. 5B). This corroborated that no overlapping of the site of stimulation occurred. Once the IPSP was evoked, the order of stimulation was inverted and the IPSP switched accordingly (Fig. 5B).
These data show that a minimum number of trials or time subject to activation is needed to trigger the underlying mechanism responsible for the DG-evoked IPSP. Since protein synthesis can be thought of as the first candidate mechanism (cf. Lamas et al. 2001; Ramírez and Gutiérrez 2001), three slices were stimulated for the whole 3-h protocol in the presence of cycloheximide, a protein synthesis blocker. Under these conditions, PP kindling stimulation potentiated DG population responses to test pulse stimulation of the PP (Fig. 4A). After completion of the 3-h protocol, DG was stimulated to evoke EPSP/IPSP sequences in pyramidal cells and GluRAs were then perfused. The GluRAs blocked all synaptic components in 21 of 21 cells tested (Fig. 4C), whereas GABAergic inhibition evoked by direct stimulation of local interneurons within CA3 was not altered (not shown).

After completing the perforant kindling stimulation and checking for the presence of GABAergic responses in CA3 to DG stimulation, three control and three PP-kindled slices taken from the same recording chamber were processed together for GAD67 immunocytochemistry. In control slices some putative inhibitory interneurons at the edge of the granular cell layer were clearly stained, whereas the granule cells of the DG had no immunoreactivity. By contrast, the stimulated slices showed clear GAD67 immunostaining in the granular cell layer and hilar region (Fig. 6).

**DISCUSSION**

This work shows a new form of synaptic plasticity produced by synaptic or direct activation of the DG in vitro that consists in the emergence of fast GABAergic transmission in the MF synapse in an activity-dependent fashion. This phenomenon coincides with the expression of GAD67 and vesicular GABA transporter (VGAT) mRNA in the granule cells and MF (Lamas et al. 2001; Ramírez and Gutiérrez 2001). With intracellular recordings of pyramidal cells of control preparations, we were unable to record synaptic potentials on blockade of glutamatergic transmission. Only after seizures (Gutiérrez 2000; Gutiérrez and Heinemann 2001) and after synaptic or direct kindling activation of the DG in vitro, monosynaptic GABAergic responses can be isolated under glutamatergic blockade. Moreover, the emergence of this inhibitory response depends on protein synthesis. Therefore this form of plasticity, demonstrated with intracellular recordings, reflects the induction of GAD67, VGAT, and GABA synthesis and the consequent emergence of GABAergic transmission in the MF synapse in response to demanding conditions. The DG-evoked fast inhibitory response has the following characteristics. 1) Its induction is of presynaptic nature since it can be obtained either by synaptic or direct activation of the DG in the presence of GluRAs. Therefore it is independent of glutamate receptor activation or of neurotransmitter-mediated cellular cascades. 2) Its induction requires a certain amount of repetitive activation. 3) It is dependent on protein synthesis. 4) It has an onset latency that parallels that of the control fast excitatory response, and it is monosynaptically driven. 5) It is mildly modulated by the group II mGluR agonist DCG-IV, but highly sensitive to the group III mGluR agonist, t-AP4. 6) A paired-pulse stimulation protocol produces facilitation of the second response, whereby presynaptic inhibition is evidenced by the activation of mGluR.

Although the present results strongly support the idea of co-release of GABA and glutamate from the MF, co-release can only be unequivocally confirmed by simultaneously recording synaptically connected granule and pyramidal cells. This technique has so far remained elusive in this system because of the very low probability of recording coupled granule and pyramidal cells. In view of this, we used a methodological approach that evidences that this phenomenon is well in agreement with responses of MF origin, and restricts possible alternative sources of the activity-dependent induced inhibitory response by taking the following into account. First, fast IPSPs can be induced after the complete pharmacological blockade of the control synaptic responses of a given cell. This ensures that no inhibitory interneurons were stimulated or that antidromic invasion of pyramidal cells could, in turn, activate inhibitory interneurons (Davenport et al. 1990; Vida and Frotscher 2000). Excitatory components arising from other glutamatergic fibers can also be ruled out since they are blocked by GluRAs, thus avoiding polysynaptic contamination (Sik et al. 1994; Weisskopf and Nicoll 1995). Moreover, the permanence of the DG-IPSP during perfusion of baclophen, which inhibits release from MF and interneurons, also supports this assumption (Brown and Johnston 1983). Second, the site and intensity of stimulation used to induce the fast IPSPs did not vary once the initial control synaptic responses were blocked, ensuring that the same fibers were stimulated. Moreover, several trials were needed to evoke this response. Interestingly, the induction of the DG-evoked GABAergic responses needed less trials when the DG was monosynthetically stimulated than when direct stimulation to a DG site was applied. This reflects that PP stimulation recruits more granule cells during the potentiation procedure than when a single MF pathway was activated. This was reflected in our experiments in which two independent MF pathways were stimulated. Third, “incomplete” kindling or “complete” kindling in the presence of the protein synthesis blocker, cycloheximide, prevents the expression of the DG-evoked GABAergic transmis-

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**FIG. 6.** Immunocytochemical determination of GAD67 in a synaptically kindled DG. A: in a slice obtained from a control rat, some interneurons are stained over the granule cell layer and hilar region. B: in kindled slices, in which the expression of GABAergic transmission in the MF-CA3 projection was verified, dense immunostaining is observed in the granule cell layer of the DG and in the hilar region. The bar represents 50 μm. ML, molecular layer; GCL, granule cell layer; H, hilus.
sion. Finally, activation of mGluR inhibits the fast inhibitory GABAergic responses in pyramidal cells on DG stimulation. It is known that the activation of mGluR reduces neurotransmitter output from MF (Manzoni et al. 1995; Min et al. 1998; Salin et al. 1996). It has also been demonstrated that inhibitory responses evoked by inhibitory cells within the DG are not sensitive to mGluR agonists (Tong et al. 1996). It has been considered that rat MF are not sensitive to L-AP4, whereas guinea pig MF are (Lanthorn et al. 1984; but see Bradley et al. 1996; Ohishi et al. 1993, 1995; Shigemoto et al. 1997). On the contrary, the group II mGluR agonist, DCG-IV, seems to selectively and almost completely depress synaptic transmission of MF origin in the rat. Interestingly, our results show that DG-evoked IPSPs are partially inhibited by DCG-IV but are practically totally inhibited by L-AP4, a group III mGluRA. It is worthy to state that these data were highly consistent from cell to cell and that we carried out the evaluation of the effects of the mGluR agonists in a blind analysis.

Three explanations can be given for the finding of simultaneous glutamatergic and GABAergic neurotransmission. One is the possibility that GABA and glutamate are co-released from the same MF. Co-release of two fast acting inhibitory neurotransmitters has already been reported to exist in the spinal cord (Jonas et al. 1998). Despite the fact that GABA and glutamate are metabolically linked, at present there are no known examples of a nerve cell that may use glutamate and GABA simultaneously as neurotransmitters. However, our data and recent evidence showing that GABAergic responses with characteristics of MF origin can be observed in pyramidal cells of CA3 (Walker et al. 2001) strongly support this hypothesis. Contrary to our data obtained in the rat, these authors found the GABAergic responses to be normally present in the juvenile guinea pig. Although marked differences are known to exist in regard to MF physiology in these two species, we have recently determined (R. Gutiérrez, unpublished observations) that DG-evoked monosynaptic GABAergic signals can be normally detected in juvenile rats (10 days old), in the absence of seizures or tetanizing the DG. Thus these data suggest that this characteristic is lost as development is completed and MF GABAergic transmission becomes dependent on activity in the adult rat. Therefore the release of GABA from MF has possibly been previously observed, but not identified as such, with experiments on juvenile rats, where the stimulation of the hilus has been used to evoke GABAergic potentials of interneuronal origin (Ben-Ari et al. 1989). Thus in these experiments MF output is likely to contribute to the inhibitory responses obtained. In light of our present results, MF-GABAergic transmission can be recognized by its inhibition by L-AP4, as we have also observed in juvenile rats.

In addition, granule cells contain trace amounts of endogenous GAD (Sandler and Smith 1991; Sloviter et al. 1996) and colocalization of GABA, and glutamate has already been proven in MF (Sandler and Smith 1991). Epileptic activity (Lehma¨nn et al. 1996; Makiura et al. 1999; Ramírez and Gutiérrez 2001; Schwarzer and Sperk 1995) and in vitro stimulation up-regulates GAD (Ramírez and Gutiérrez 2001) and GABA synthesis (this work and G. Gómez-Lira, E. Trillo, M. Ramírez, M. Asai, M. Sitges, and R. Gutiérrez, unpublished observations) and the VGAT mRNA expression (Lamas et al. 2001). In view of the aforementioned, it is reasonable to state that granule cells have the necessary machinery for the synthesis, vesiculation, and release of GABA and that protein synthesis is required to trigger the GABA-releasing process. Indeed, we have found that inhibition of protein synthesis with cycloheximide prevents the expression of inhibitory transmission from MF, whereas inhibitory transmission from the interneuronal population onto pyramidal cells in CA3 remains intact. This also confirms the presynaptic nature of the phenomenon. Moreover, pyramidal cell responses to GABAergic signals from MF origin seem possible since it has been shown in cultured pyramidal hippocampal cells, that GABA receptors cluster apposed to mismatched glutamatergic presynaptic elements, even in the absence of GABA input, suggesting the hypothesis that there is an element common to GABA and glutamate synapses (Rao et al. 2000). Whether these clusters are present in slices acutely prepared from adult animals is not known, and this is currently under investigation. From our data, it seems likely that pyramidal cells do normally have GABA receptors in apposition to MF inputs, or that they can express them under certain conditions. The similar latencies of the DG-evoked IPSP and control EPSP and the fact that the IPSP’s reversal potential can be readily obtained is consistent with the presence of GABA receptors close to the soma, where MF impinge onto pyramidal cells. Effective inhibition of the MF input to proximal dendrites of CA3 pyramidal cells can only take place if they are located close to the soma (Miles et al. 1996; Soltesz et al. 1995; Urban and Barrionuevo 1998).

A second explanation implies the possible activation of silent MF GABAergic synapses (Poisbeau et al. 1997), whereby MF may release GABA from specialized synaptic contacts, which are activated by the kindling protocol. Compartmentalization of MF has recently been documented (Maccaferri et al. 1998), thus different synaptic terminals of a common output pathway can be specialized and selectively activated depending on the excitation/inhibition balance of the system. However, recent evidence of our laboratory (M. Vivar, H. Romo-Parrá, and R. Gutiérrez, unpublished observations) has shown that both pyramidal cells and intrinsic interneurons within CA3, despite receiving different types of MF contacts, respond with a GABAergic potential on DG stimulation after seizures. From the present results it is not possible to determine whether GABA and glutamate are released from the same or different terminals. It is suggestive that there seems to be a selective regulation of MF-GABA release by group III mGluR, which can give support to this idea. Conversely, a link of group II mGluR to the GABA-releasing machinery, and of group II mGluR to the glutamate-releasing machinery can be suggested. Indeed, mGluR II activation produces a downregulation of the exocytotic machinery (Kamiya and Ozawa 1999), therefore not only an inhibition of Ca2+ entrance explains inhibition of neurotransmitter release. Thus this can link mGluR to the different machineries of release (GABA and glutamate) rather than to a general inhibition of release by Ca2+-dependent processes. In that way, spillover of glutamate may presynaptically activate group II mGluR to hamper glutamate release. As excitability rises, GABAergic transmission is expressed, and further excitation and release of glutamate may then activate group III mGluR. How the interplay of these possibilities is orchestrated is a matter of future investigations.

The mossy fibers of the rat are immunolabeled against
mGluR2, mGluR7a, and mGluR7b (Bradley et al. 1996; Shigemoto et al. 1997), and human MF possess mGluR2/3 and 4 (Blumcke et al. 1996; Lie et al. 2000). Electron microscopy has revealed the spatial segregation of group II and group III mGluRs within presynaptic elements, where immunolabeling for the group III receptors was predominantly observed in presynaptic active zones of asymmetrical and symmetrical synapses, whereas that for the group II receptor (mGluR2) was found in preterminal rather than terminal portions of axons (Shigemoto et al. 1997). Furthermore, the granule cells of the DG express groups II/III mGluR mRNA (Ohishi et al. 1993, 1995). Target cell–specific segregation of receptors was shown for group III mGluRs, suggesting that transmitter release is differentially regulated by 2-amino-4-phosphonobutryrate–sensitive mGluRs in individual synapses on single axons according to the identity of postsynaptic neurons (Shigemoto et al. 1997). Our evidence shows for the first time with physiological recordings that in the rat this segregation is also true for presynaptic elements, which can be differentially modulated by one type of receptor or another according to the class of neurotransmitter released from a given terminal. The fact that group III mGluR are found in symmetrical as well as asymmetrical synapses and group II mGluR are found in preterminal rather than in terminal portions of the axons (Shigemoto et al. 1997) is in accordance to the type of modulation that 1-AP4 and DCG-IV exert on MF GABAergic and glutamatergic transmission, respectively. A recent report, compatible with the idea of a differential presynaptic modulation of MF glutamate and GABA release has shown that the granule cells express mGluR4 after seizures (Lie et al. 2000), which coincides with the appearance of DG-evoked GABAergic transmission and 1-AP4 modulation after seizures (Gutiérrez 2000).

A third explanation for the appearance of the kindled-induced IPSP in CA3 is the direct activation of inhibitory interneurons in the DG that can project to CA3. However, as discussed earlier, the experimental design used in this work makes the possibility of DG interneuron-to-pyramidal cell activation unlikely.

We have found that the DG-evoked IPSP undergoes presynaptic inhibition by activation of mGlu receptors, with a higher sensitivity to activation of group III receptors; that it persists in the presence of a GABAergic agonist, that together with GluRAs avoids polysynaptic contamination; that it undergoes paired-pulse potentiation, which is enhanced by DCG-IV and 1-AP4; avoids polysynaptic contamination; that it undergoes paired-pulse inhibition blocks maintenance but not induction of epileptogenesis in hippocampal slice. Brain Res 599: 338–344, 1992.


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