The Expression of GABA in Mossy Fiber Synaptosomes Coincides with the Seizure-Induced Expression of GABAergic Transmission in the Mossy Fiber Synapse

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Although the granule cells of the dentate gyrus are glutamatergic, they contain the machinery for the synthesis and vesiculation of GABA. Furthermore, glutamic acid decarboxylase and the vesicular GABA transporter mRNA are expressed in the granule cells and mossy fibers in an activity-dependent manner, suggesting that these cells release GABA in addition to glutamate. Supporting this hypothesis, we found that seizures induce simultaneous glutamatergic and GABAergic transmission in the mossy fiber projection. To further explore this expression of inhibition, we looked for the presence and expression of endogenous GABA in a synaptosomal preparation enriched with mossy fiber nerve endings of kindled rats. We also studied the capacity of this preparation to capture and release [3H]GABA under control conditions and after kindling epilepsy. In accordance with our hypothesis we show that the mossy fiber synaptosomal preparation of the kindled rats has a significantly higher content of endogenous GABA than controls. We also found that the protein content in the mossy fiber synaptosomal preparation of kindled rats was significantly augmented, which is consistent with mossy fiber sprouting. Due to this, the total [3H]GABA incorporated in the synaptosomal preparation was also augmented. However, [3H]GABA uptake (expressed in % radioactivity/mg protein) and its evoked release were similar in both groups. With the present results, we provide further support for the hypothesis of the emergence of GABAergic transmission in the mossy fiber synapse that can constitute a protective mechanism in response to seizures.

Key Words: GABA; glutamate; mossy fibers; kindling; epilepsy; synaptosomes; rat; inhibition.

INTRODUCTION

Although the granule cells of the dentate gyrus (DG) are glutamatergic (6), they normally contain glutamic acid decarboxylase (GAD67; 31, 34). Long-lasting electrical stimulation enhances the concentration of this enzyme in these cells (34, 11) and, after kainic acid-induced limbic seizures, they transiently express mRNA for GAD, and GABA can be immunocytochemically detected (32, 17, 20, 33). This evidence suggests that mossy fibers are able to synthesize and release GABA. Moreover, an origin for GABA, other than the GAD-mediated synthetic pathway, has been proposed to exist particularly in mossy fiber terminals (MFT; 38). In line with this, we showed for the first time that granule cells and, with the use of this preparation, mossy fiber terminals express the vesicular GABA transporter (VGAT) mRNA and that kindling epilepsy, acute seizures, and synaptic potentiation in the absence of epileptic activity upregulate this expression (16). Furthermore, we presented for the first time electrophysiological evidence showing that seizures (10, 12) and synaptic potentiation (11) induce functionally relevant simultaneous glutamatergic and GABAergic transmission in the mossy fiber projection in the rat; this simultaneous transmission has also been observed in healthy young guinea pig preparations (41).

The monosynaptic nature of this fast inhibitory response has been established, and the possibility that this aberrant inhibitory response in CA3 pyramidal cells to DG stimulation, during glutamatergic transmission blockade, is due to stimulation of sprouted interneurons (7), or to the recruitment of inhibitory interneurons through electrical synapses, has been ruled out (10). However, another possibility worth tak-
ing into account is that GABA supply in the mossy fibers might come from the extracellular space through an enhanced uptake mechanism in seizing rats. Constitutive release, on the other hand, can take place only if granule cells synthesize this inhibitory neurotransmitter, and this possibility seems likely to occur after seizures (33). Therefore, our goals in this work were to induce the aberrant inhibitory response in CA3 to DG stimulation during glutamatergic transmission blockade, with the kindling model of epilepsy. With a synaptosomal preparation enriched with mossy fiber terminals (39), we looked for the presence of endogenous GABA in control animals and whether there were differences in its content in kindled animals. Furthermore, we assessed the ability of MFT to incorporate and release \([{}^1H\text{GABA}. \text{Consistent with our hypothesis, kindling induced fast inhibitory responses in CA3 to DG stimulation after blocking glutamatergic transmission. By measuring endogenous amino acid neurotransmitters in the MFT, we found that the concentration of GABA in kindled preparations was significantly higher than that in control preparations, but no changes in exogenous GABA uptake and release were observed between the control and the kindled groups.

METHODS

Kindling procedure. Male Wistar rats (230–250 g) were anesthetized with ketamine (60 mg/kg ip) and implanted with bipolar stainless steel electrodes (80 KΩ) into the left basolateral amygdala (AP 2.5, L 5, H 8.5; 22). Starting 7 to 10 days after the operation, the animals were daily stimulated with a 1-s train of square pulses (0.1-ms pulse duration, 60 Hz, 500 μA) until five generalized convulsive seizures (stage V; 24) were evoked. All experimental procedures were approved by the Committee on Ethical Animal Research of our institution and of the Ministry of Health.

In vitro intracellular recordings. Twenty-four hours after the last kindled seizure, we prepared hippocampal slices from a rat belonging to the kindled group to be used for the neurochemical experiments on a given day. The rats were decapitated under deep ether anesthesia, brains were 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, as previously reported (10, 12). Slices were then transferred to an interface recording chamber and were constantly perfused with oxygenated ACSF at 35°C containing (in mM) NaCl, 124; KCl, 3; NaH2PO4, 1.25; MgSO4, 2; CaCl2, 2; NaHCO3, 26; glucose 10; pH 7.35. The drugs used were diluted in the ACSF, namely, (S)-2-amino-5-phosphonovaleric acid (APV; 30 μM; Tocris); 6-nitro-7-sulfamoylbenzoflavonolazine-2,3-dione (NBQX; 10 μM; Tocris), and bicuculline methiodide (20 μM; Sigma) and atropine (5 μM; Sigma).

An hour after incubation, intracellular activity of pyramidal cells located in CA3, close to region CA2, was recorded with microelectrodes pulled with a Brown–Flaming horizontal puller with a resistance of 70–90 MΩ and filled with 2 M potassium acetate, using an AxoClamp 2B amplifier. Acquisition and offline analysis were done with the program pClamp6 (Axon Instruments). For electrical stimulation, a bipolar glass-insulated platinum wire (50 μm) electrode was placed over the granular cell layer of the DG. Pulses had a duration of 0.1 ms and intensity was fixed to a value that evoked 70% of the excitatory postsynaptic potential (EPSP) amplitude needed to reach threshold for evoking action potentials.

Mossy fiber synaptosome preparation. Mossy fiber synaptosomes were prepared as previously described (5, 16, 37–39). Briefly, adult control (n = 85) and kindled (n = 25) male Wistar rats (200–250 g) were killed by decapitation 24 h after the last seizure and the brains were rapidly removed. For each observation, the hippocampi of two rats were pooled, dissected on ice, and homogenized in 5 mM Tris–HCl buffer (pH 7.4) containing 0.32 M sucrose and 1 mM MgCl2 (1 g of tissue/10 ml of buffer) with a homogenizer (reostate type 3PN1010; Staco Energy Products Co., and Craftsman pestle Model 255812) by six up-and-down movements. The homogenate was centrifuged at 900 g for 10 min (at 4°C) in a SM-24 rotor (Sorvall RC 285; Dupont). The pellet, which contains a mixture of synaptosomes, was washed once by resuspension in buffer and centrifuged again. The pellet (P) was then resuspended and layered on three-step Percoll gradients, 10, 15, and 23% in 0.32 M sucrose and 1 mM MgCl2 (pH 7.4) (1 ml of P/2 ml of Percoll), and centrifuged at 49,000 g for 15 min (4°C). After centrifugation, we obtained eight bands (37). The synaptosomal fraction PII was collected from the 10–15% Percoll interface and PIII from the 15–23% Percoll interface, as previously described (37–39). Aliquots of 40 μl were collected from each fraction to determine protein concentration by the method of Lowry (19).

Endogenous amino acid determination. We determined the total amount of endogenous amino acids in fraction PIII from four kindled and three control rats. Samples (650 to 780 μl) from the synaptosomal fraction PIII of each rat were collected and transferred to separate tubes. An aliquot of a concentrated solution of perchloric acid (PCA)/ethylenediamine tetaacetic acid (EDTA) (Suprapur; Merck Laboratories, Germany) was added to each tube to give a final concentration of 0.1 M/0.1 mM, respectively. The synaptosomal suspensions were vortex mixed and centrifuged. The supernatants resulting from this centrifugation were stored at −40°C for later analysis. In order to standardize neurotransmitter concentrations per milligram of synap-
tososomal protein, the pellets resulting from the centrifuga-
tion were suspended in NaOH 0.1 N and used for
protein determination by the method of Lowry (19).
The samples containing the amino acid neurotransmit-
ters were injected into the HPLC system within the
next 2 days after obtainment.

Ten microliters of sample suspended in 0.1 M PCA/
0.1 mM EDTA were mixed with 20 μl of the ophthal-
aldoxy reagent solution (OPA; Pierce, Rockford, IL).
After 120 s (optimum derivatization time for the reac-
tion) a 10-μl aliquot was injected into the HPLC sys-
tem. An analytical column (Nova-Pak C-18; 75 ×
3.9-mm internal diameter, particle size 10 μm) set at
24°C and a fluorescence detector set at 360 nm (exci-
tation wavelength) and at 450 nm (emission wave-
length) were used. A linear gradient elution program
performed over 30 min was applied for amino acid
eution; eluent A (30 mM sodium acetate buffer, pH
6.8), from 100 to 50%, and eluent B (methanol; Caledon
Laboratories Ltd., Georgetown, Ontario, Canada),
from 0 to 50%, at a flow rate of 1 ml/min. The concentra-
tions of glutamate, aspartate, glutamine, and
GABA in the experimental samples were calculated
with calibration curves obtained from the injection,
into the HPLC system, of increasing concentrations of
standard amino acid mixtures (Sigma, St. Louis, MO)
after OPA derivatization. Student’s t test was used for
statistical evaluations. A P < 0.05 was considered
statistically significant.

dynorphin detection by radioimmunoassay. Release
experiments were conducted as described below. Perfu-
sates were collected in assay tubes with 500 μl HCl (0.1
N). They were then incubated at 42°C for 15 min and
frozen. For dynorphin 1-8 determination (IR-Dyn), the
samples were purified through an Amberlite XAD-2 col-
lumn, rinsed with 20 ml HCl (0.1 N) and 40 ml H2O. The
samples were then diluted in 20 ml methanol. After evap-
oration, the samples were resuspended in 1 ml distilled,
deionized water. Radioimmunoassay was carried out on
samples of 100 μl in triplicate with a polyclonal antibody
against dynorphin 1-8 obtained in our laboratory. Our
antibody displayed a cross-reactivity of 100% with dynor-
phin 1-8 and negligible cross-reactivity against the
following peptides (in %): Met-enkephalin, 0.76; Leu-en-
kephalin, 2.9; Met-enkephalin-Arg, 0.01; Leu-enkephalin-
Arg, <0.01; Met-enkephalin-Arg-Phe, <0.01; Met-en-
kephalin-Arg-Gly-Leu, <0.01; α-endorphin, <0.01;
β-endorphin, <0.01; γ-endorphin, <0.01.

[3H]GABA uptake and release experiments. Uptake
and release of [3H]GABA were studied by the superfus-
tion technique previously described (35, 36) with some
modifications. Briefly, final synaptosomal fractions PII
and PIII (800 μl) were resuspended in 1 ml of Krebs-
Ringer medium (composition in mM: NaCl, 119; KCl,
4.3; CaCl2, 1.25; KH2PO4, 0.85; MgSO4, 0.84; NaHCO3,
24.8; glucose, 10; pH 7.4) and incubated with

\[ ^{3} \text{H} \text{GABA} \]
Kindling-induced changes in the amino acid content of mossy fiber nerve endings. (A) Bars represent the mean content (% of the total content of amino acids ± SEM) of glutamate, aspartate, GABA, and glutamine in mossy fiber synaptosomes from three control (white bars) and four kindled (black bars) rats. Total amino acid neurotransmitters refers to the sum of the concentrations of glutamate, aspartate, GABA, and glutamine in synaptosomes from each animal. Asterisks indicate a significant statistical difference (Student t test; P < 0.05). (B) Chromatogram of standards. Peaks from a mixture of Glu, Asp, glutamine (Gln), and GABA. OPA, OPA reagent peak. The volume injected (10 μl) contains 7.3 ng of each amino acid. Retention times, in minutes, are indicated in parentheses below the name of each molecule. Chromatograms of (C) a control and of (D) a kindled sample.
beta-alanine, were effective in preventing [3H]GABA uptake in both control and kindled preparations. Niptocotic acid (at 1 and 10 mM) completely blocked [3H]GABA uptake in fractions PII and PIII from both groups, whereas beta-alanine (at 1, 5, and 10 mM) was ineffective (not shown).

DISCUSSION

Our electrophysiological and neurochemical data further support the hypothesis that the granule cells of the DG are able to synthesize and release GABA in response to seizures, as previously suggested by immu-

TABLE 2

Values of the Protein Content, [3H]GABA Content per Sample and per Protein Volume, and Evoked [3H]GABA Released in and from the Synaptosomal Mossy Fiber Preparations from Control and Kindled Rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Kindled</th>
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<tbody>
<tr>
<td></td>
<td>PII</td>
<td>PIII</td>
</tr>
<tr>
<td>Protein (mg/ml ± SEM)</td>
<td>1.77 ± 0.28 (n = 15)</td>
<td>2.08 ± 0.49 (n = 15)</td>
</tr>
<tr>
<td>[3H]GABA content (%/sample)</td>
<td>17.66 ± 1.3 (n = 10)</td>
<td>20.5 ± 1.2 (n = 12)</td>
</tr>
<tr>
<td>[3H]GABA uptake (% [3H]GABA/mg prot)</td>
<td>6.85 ± 1.25 (n = 10)</td>
<td>6.64 ± 1.05 (n = 12)</td>
</tr>
<tr>
<td>Evoked [3H]GABA release</td>
<td>21.49 ± 8.7 (n = 10)</td>
<td>26 ± 10.6 (n = 10)</td>
</tr>
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Note. n is the number of rats, 2 rats were used for each determination.

* Significant at P < 0.05 vs PII control (Student t test).

** Significant at P < 0.05 vs PII kindled and PIII control (Student t test).
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nocytochemical (17, 20, 26, 31–34), neurochemical (37, 38), molecular (16), and electrophysiological evidence (10–12). Moreover, the significant enhancement of the content of GABA in the MFT of the kindled animals, together with our previous evidence showing the expression of GAD and VGAT mRNA in granule cells and mossy fibers (26, 16), indicates that the releasable GABA in the mossy fiber synapse originates from constitutive sources.

Previous reports show some GAD$_{67}$ staining in the DG of control preparations (31, 34). However, electrophysiological recordings show no evidence of GABAergic transmission in the mossy fiber pathway after glutamatergic transmission was blocked in healthy rats. However, after kindling in vivo and in vitro, in addition to simultaneous glutamatergic and GABAergic transmission from the mossy fibers, a dense immunostaining in the DG and mossy fibers has been found (11, 17, 26, 32, 34). This evidence implies that GABA can be synthesized in granule cells and that it must be present in MFT (20) and related to synaptic vesicles (34). Indeed, with the use of the MFT preparation, and in agreement with other authors (37), we found that MFT contain GABA and we extend this observation by establishing that its content is significantly enhanced in kindled animals. These observations parallel the appearance of inhibitory transmission upon pyramidal cells, under glutamate receptor blockade. Moreover, this GABAergic response is inhibited by metabotropic glutamate and GABA$_A$ receptor agonists (10, 11) that depress synaptic transmission from mossy fibers (2, 30). Together, these data strongly suggest that the mossy fibers are indeed enabled to release GABA.

Although the content of mossy fiber terminals in this synaptosomal preparation has been well established, and we show that it releases dynorphin, other inhibitory processes in the preparation can be responsible for the presence of GABA. However, in favor of the specificity of our preparation, we show that kindling induces a significant selective enhancement of protein concentration and release of dynorphin in and from fraction PIII and not fraction PII. This is in agreement with mossy fiber sprouting. Interestingly, colchicine lesions of the dentate gyrus produce the opposite effect, namely, a decrease in the protein content and release of dynorphin in the MFT preparation (5). This is relevant considering the well-documented phenomenon of sprouting of mossy fibers in response to epilepsy (1, 3, 14). It is noteworthy to point out our high number of experiments and highly reproducible results in this regard. Also, that our data show a low concentration of glutamine in fraction PIII speaks in favor of the high content of synaptosomes and low glial contamination in the preparation.

An altered function of the GABA uptake/release mechanism of epileptic patients and kindled rats has been described (8), in which a decreased release of GABA by the electrogenic outward transport of the GAT-1 is observed. In our experiments with [H]$^3$GABA, however, uptake and release seem to be unaffected, at least in the MFT. Although this MFT preparation has been well characterized (5, 37–39, 18), we cannot fully discard that related contaminant processes can be embedded in the synapses and that they may contribute to possible obscure uptake changes. Nonetheless, we can propose that the uptake is not the source for GABA in the MF, rather, constitutive GABA can be released from the sprouted mossy fibers as a compensatory mechanism that can act to avoid further spread of hyperexcitation in the hippocampus after epilepsy. This is supported by the up-regulation of GAD (26) and of the VGAT mRNA (16) and, finally, the appearance of GABAergic transmission after kindled seizures (10, 12) and after induction of hyperexcitability in vitro (11).

The presence of GABA transporters is restricted to neurons that synthesize and release GABA and glial cells (15, 25, 29). Some GABAergic cells, immunocytochemically characterized by the presence of GAD$_{67}$ or GABA, do not contain or contain traces of GAT-1, respectively, but not vice versa (28). In line with this, it has been reported that MFT release GABA (37) and that GAT-1 is present in both principal cells and interneurons of the hippocampus (9, 29). Therefore, since MFT from control animals capture GABA and nipeotic acid blocks this uptake, it is reasonable to propose that they normally have the GABA transporter (GAT-1), indicating that these cells do have the ability to release constitutive GABA, i.e., that they are actually potentially GABAergic. This is supported by the finding of overexpression of GAD$_{67}$ by our group and others (17, 32, 33). Moreover, Taupin et al. (37) have hypothesized that the synthesis of GABA in mossy fibers is independent of GAD$_{67}$. Although we and others have found expression of GAD$_{67}$ in granule cells and mossy fibers after seizures, we do not exclude that some of the GABA present in the mossy fibers can originate from an alternative route. Glutamate is closely linked metabolically to both GABA and aspartate, as well as to its amide, glutamine. Interestingly, we found that the content of endogenous aspartate is diminished in the same proportion in which endogenous GABA was augmented, without changes in the content of glutamate. This possibility, however, has to be further explored.

In light of the results presented in this work and those previously reported (10–12), we propose that the inhibitory phenotype of granule cells, i.e., transient expression of GAD$_{67}$ and synthesis and release of GABA, is functionally expressed only after the establishment of enhanced excitability (11), a notion that is gaining increasing support (20). Two missing links in our hypothesis were the apparent lack of the VGAT in the mossy fibers and the presence of GABA$_A$ receptors
in glutamtergic synapses. Indeed, Chaudhry et al. (4) were unable to detect it with immunocytochemical techniques in mossy fibers of normal rats. However, recent evidence from our laboratory (16) shows that VGAT mRNA is indeed present in the DG and MFT of seizing animals and that its expression is regulated by activity, suggesting that this transporter can be synthesized in mossy fibers. On the other hand, it has been recently shown in cultured pyramidal hippocampal cells that GABA_A receptors cluster on pyramidal cells in the absence of GABA input and were even apposed to mismatched glutamergic presynaptic elements. The existence of such mismatched appositions suggests the hypothesis that there is an element common to GABA and glutamate synapses (27). It is therefore likely that pyramidal cells do have GABA receptors in apposition to mossy fiber inputs or that they can express them under certain conditions. Indeed, DG stimulation is able to induce fast IPSPs in the pyramidal cells, whose onset latency parallels that of the EPSP and whose reversal potential can be easily obtained (10–12; see also 41). The onset latency of the control EPSP coincides with previous reports (2) and the latencies of the DG-evoked EPSP and IPSP are similar, even after the probability of neurotransmitter release is reduced. This points to a somatic or proximal dendritic location of the inhibitory input (21).

We conclude that kindling epilepsy results in the expression of fast inhibition in the DG–CA3 synapse due to the release of constitutive GABA. Moreover, in support of this hypothesis, we have recently found that the inhibitory neurotransmission from mossy fibers also acts upon inhibitory interneurons of CA3 (C. Vivar, H. Romo-Parra, and R. Gutiérrez, unpublished observations). The transient overexpression of GAD67 (17, 26, 32) and GABA synthesis in granule cells and the consequent inhibitory transmission upon pyramidal cells of CA3 (10, 12) are likely to serve as a protective mechanism after the establishment of epilepsy, whereby further spread of epileptic activity can be limited. Indeed, direct inhibition of pyramidal cells, and possibly collateral presynaptic inhibition of other mossy fibers by activation of GABA_A receptors, serving the inhibitory modulation of GABA and glutamate in this synapse (2, 40), can be involved in the postictal depression and inhibition (13, 23) and in the alterations of information storage in the hippocampus that follow seizures.

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REFERENCES


