Differential Epilepsy-Associated Alterations in Postsynaptic GABA<sub>A</sub> Receptor Function in Dentate Granule and CA1 Neurons

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Gibbs, John W., III, Melissa D. Shumate, and Douglas A. Coulter. Differential epilepsy-associated alterations in postsynaptic GABA<sub>A</sub> receptor function in dentate granule and CA1 neurons. *J. Neurophysiol.* 77: 1924–1938, 1997. Alterations in GABAergic function associated with the development of temporal lobe epilepsy (TLE) were examined with the use of patch-clamp recording techniques in dentate granule (DG) and CA1 neurons acutely isolated from control and spontaneously epileptic rats in which TLE was elicited by pilocarpine injection 3–17 wk before use. The maximal efficacy of γ-aminobutyric acid (GABA) in activating whole cell GABA currents increased significantly in epileptic DG neurons relative to controls. This efficacy increase was due to a 78% enhancement in the functional capacitance-normalized GABA<sub>A</sub> receptor (GABA<sub>A,R</sub>) current density in epileptic DG neurons. Increased DG GABA<sub>A,R</sub> current density was not accompanied by alterations in GABA potency (EC<sub>50</sub>). However, the maximal sensitivity of GABA-evoked currents to blockade by zinc increased 190% in epileptic neurons. Augmentation of epileptic DG neuron GABA-evoked currents by the broad-spectrum anticonvulsant benzodiazepine clonazepam (100 nM) was enhanced 114% relative to controls, whereas augmentation by the benzodiazepine, (BZ<sub>1</sub>)-selective agonist zolpidem (100 nM) was decreased by 66%. In contrast to DG neurons, maximal efficacy of GABA in activating GABA currents decreased in epileptic CA1 neurons relative to controls, due to a 52% decrease in functional capacitance-normalized GABA<sub>A,R</sub> current density. This altered efficacy of GABA was accompanied by an increased GABA potency (GABA EC<sub>50</sub> was 35.8 and 24.5 μM in control and epileptic neurons, respectively). Sensitivity of GABA-evoked currents to blockade by zinc was unchanged in epileptic CA1 neurons, whereas clonazepam (100 nM) augmentation of CA1 GABA-evoked currents decreased 81% relative to controls. These regionally distinct epilepsy-associated modifications in hippocampal GABAergic function may be due to discrete structural alterations in postsynaptic GABA<sub>A,R</sub>s accompanying epileptogenesis, could be therapeutically important, and undoubtedly could contribute to the enhanced limbic excitability underlying TLE.

INTRODUCTION

The hippocampal formation is integrally involved in the generation of partial seizures underlying temporal lobe epilepsy (TLE). The factors involved in triggering these recurrent spontaneous seizures within the limbic system are a subject of active investigation. There are characteristic patterns of pathology evident in hippocampal tissue resected from patients with chronic TLE (reviewed in Babb and Pretorius 1993). This pattern of pathology has been termed mesial temporal sclerosis. Considerable attention has been paid to these anatomic correlates of TLE, with detailed examinations of the patterns and nature of neuronal loss and circuit rearrangements accompanying development of TLE being a focus of vigorous research both in surgically resected tissue from TLE patients (Babb et al. 1991; Houser et al. 1990; Sutula et al. 1989) and in animal models of TLE (Mello et al. 1993; Sutula et al. 1989; Tauck and Nadler 1985). The role of these anatomic changes in the generation of limbic seizure susceptibility is an area of lively debate. However, somewhat lost in this anatomic debate is the potential epileptogenic role played by functional alterations in postsynaptic intrinsic properties and excitatory and inhibitory neurotransmitter receptor expression patterns that may occur in the surviving neurons in epileptic hippocampus.

Alterations in neurotransmitter-mediated responses have been described in various hippocampal neuronal populations both as acute consequences of limbic seizure activity and as a chronic epilepsy-associated change in animals with TLE. In physiological studies, excitatory synaptic responses mediated through activation of N-methyl-D-aspartate receptors are altered as a consequence of kindling in dentate granule (DG) cells (Köhr and Mody 1994; Mody and Heineman 1987), and after the development of TLE in both CA1 and DG in the “super-kindling” model of Lothman et al. (1995). In addition, the binding properties of N-methyl-D-aspartate receptors in CA3 neurons are altered as a long-term consequence of kindling (Martin et al. 1992).

Inhibitory synaptic responses mediated through activation of γ-aminobutyric acid-A receptors (GABA<sub>A,R</sub>s) have also been shown to be altered in CA1 and dentate gyrus by development of an epileptic condition. Paired pulse depression was enhanced by kindling (deJonge and Racine 1987; Oliver and Miller 1985), and miniature inhibitory postsynaptic currents (mIPSCs) were significantly larger in the dentate gyrus of kindled animals relative to controls (Otis et al. 1994). In binding studies, both [<sup>3</sup>H]muscimol binding and muscimol-stimulated synaptosomal Cl<sup>−</sup> uptake were significantly enhanced in the dentate gyrus of kindled animals and reduced in CA1 relative to controls (Titulaer et al. 1994, 1995).

In the present study, potential functional epilepsy-associated alterations in the physiological and pharmacological properties of GABA<sub>A,R</sub>s were examined with the use of whole cell patch-clamp recordings from hippocampal CA1 and DG neurons isolated from rats in which a TLE condition was induced by pilocarpine injection an average of 6–7 wk before use.
METHODS

Injections

The protocol for pilocarpine injections used in this study was adapted from Mello et al. (1993). Adult rats, ~120 days postnatal, were kept in a climate-controlled (70°F) room on a 12-h light:dark cycle and provided with food and water ad libitum 1 wk before injection. Rats were injected first with scopolamine methyl nitrate (1 mg/kg ip) to minimize the peripheral effects of pilocarpine, and were subsequently injected 30 min later with pilocarpine (350 mg/kg ip). The pilocarpine injection usually triggered continuous seizures reminiscent of status epilepticus (SE): long-duration (>30 min) epileptic attacks within 10–30 min after pilocarpine injection. Rats that did not exhibit class 3 behavioral seizures after 1 h post pilocarpine injection were injected with a second dose of pilocarpine (175 mg/kg ip). Diazepam (4 mg/kg ip) in 50% propylene glycol was administered 1 h after the onset of SE to stop seizure activity. Additional diazepam was administered at 3 and 5 h post onset of SE as needed. Pilocarpine-induced SE usually took 3–5 h to terminate. To facilitate survival, weak rats were given an oral mixture of sucrose and powdered milk for several days after pilocarpine-induced SE. Control rats \( (n = 14) \) were treated in exactly the same way as pilocarpine-injected rats \( (n = 13) \), except that a subconvulsive dose of pilocarpine (35 mg/kg) was administered. Additionally, two-aged-matched naive animals without pilocarpine injections were also used as controls, and showed experimental results not different from subconvulsive treated rats. Therefore results from both subconvulsive and naive controls were pooled.

Epilepsy diagnosis

Beginning 2wk after the pilocarpine injection, rats were video monitored for 8 h/day until at least one spontaneous seizure was observed. Seizures consisted at least of class 3 or higher activity 120 NaCl, 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 25 glucose, and 20 piperazine-\(\text{N},\text{N}\)-2-hydroxy-\(\text{N}\)-ethane sulfonic acid (HEPES) medium composed of (in mM) 155 NaCl, 3 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 26 NaHCO\(_3\), and 10 dextrose. Hippocampal slices (450 \(\mu\)m) were cut on a 12° agar ramp with a vibratome (Rafiq et al. 1993, 1995) and incubated for 1 h in an oxygenated medium containing (in mM) 120 NaCl, 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 25 glucose, and 20 piperrazine-\(\text{N},\text{N}\)-bis[2-ethanesulfonic acid] (PIPES), pH adjusted to 7.0 with NaOH at 32°C. Slices were enzymed in 3 mg/ml Sigma protease XXIII in PIPES, thoroughly rinsed, and incubated another 30 min in PIPES medium before dissociation. The dentate gyrus and CA1 of the hippocampus were visualized with the use of a dark-field microscope and dissected out. Chunks (1 mm\(^3\)) were cut from each area, and cells were then mechanically dissociated by trituration of the chunks through a series of Pasteur pipettes of decreasing bore sizes. The resulting cell suspension was then plated onto gelatin-coated slides, and stored at ~70°C. Sections were then thawed, hydrated, and developed in Timm’s stain (Haug 1973). Slide-mounted sections were then dehydrated, placed in xylene, and coverslipped for histological examination.

Timm’s stain scoring

The scoring for the degree of mossy fiber sprouting into the supragranular layer of the dentate gyrus was determined blindly (i.e., by an investigator who did not know the seizure history of the animal) as described previously (Tauc and Nadler 1985). The Timm’s scores of 3 sections per animal were averaged for a final score for each animal. Mossy fibers were identified by black staining for zinc-containing synaptic terminals (e.g., Fig. 6). Sections with no or occasional supragranular dentate staining were given a score of 0. Sections with scattered Timm’s stain over all areas of the DG layer were scored 1. Sections that showed sparse staining interspersed with regions of heavier staining or a continuous band of intermediate staining in intensity between sections scored 1 and 3 were given a score of 2. Sections with a dense, continuous band of stain were scored 3 (Tauc and Nadler 1985). Significance was calculated by the Mann-Whitney rank sum test.

Acute isolation of neurons

Experiments were conducted on neurons acutely isolated from adult rat hippocampus with the use of methods that have been previously described (Gibbs et al. 1996a,c; Oh et al. 1995). The brain was dissected and placed in a 4°C chilled, oxygenated (95% \(\text{O}_2\)-5% \(\text{CO}_2\)) artificial cerebrospinal fluid solution composed of (in mM) 201 sucrose, 3 KCl, 1.25 NaHPO\(_4\), 2 MgCl\(_2\), 2 CaCl\(_2\), 26 NaHCO\(_3\), and 10 dextrose. Hippocampal slices (450 \(\mu\)m) were cut on a 12° agar ramp with a vibratome (Rafiq et al. 1993, 1995) and incubated for 1 h in an oxygenated medium containing (in mM) 120 NaCl, 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 25 glucose, and 20 piperrazine-\(\text{N},\text{N}\)-bis[2-ethanesulfonic acid] (PIPES), pH adjusted to 7.0 with NaOH at 32°C. Slices were enzymed in 3 mg/ml Sigma protease XXIII in PIPES, thoroughly rinsed, and incubated another 30 min in PIPES medium before dissociation. The dentate gyrus and CA1 of the hippocampus were visualized with the use of a dark-field microscope and dissected out. Chunks (1 mm\(^3\)) were cut from each area, and cells were then mechanically dissociated by trituration of the chunks through a series of Pasteur pipettes of decreasing bore sizes. The resulting cell suspension was then plated onto 35-mm culture dishes in \(N\)-2-hydroxy-ethylpiperazine-\(\text{N},\text{N},\text{N}\)-tetraacetic acid, 2 MgCl\(_2\), and 0.5 CaCl\(_2\), pH adjusted to 7.35 with NaOH. A 4-nM junction potential was measured between this solution and the bath solution. All voltages were corrected accordingly. Whole cell patch-clamp recording experiments were conducted on a Nikon inverted microscope equipped with Hoffman modulation contrast optics. Electrodes (4–8 M\(\Omega\)) were pulled on a Narishige PP-83 microelectrode two-stage puller with the use of thin-walled borosilicate capillary glass (WPI, Sarasota, FL). The pipette solution also contained an intracellular ATP reconstitution consisting of 10 mM Mg\(^{2+}\)-ATP and 22 mM phosphocreatinine. The intracellular ATP maintenance solution was used to fill the shank of the electrode, but was omitted from the solution that was used to backfill the tip of the electrode.

Voltage-clamp recordings in isolated neurons

The intracellular (pipette) solution contained (in mM) 10 Trizma phosphate (dibasic), 28 Trizma base, 11 ethylene glycol bis-(\(\beta\)-aminoethyl ether)-\(\text{N},\text{N},\text{N}\)-tetraacetic acid, 2 MgCl\(_2\), and 0.5 CaCl\(_2\), pH adjusted to 7.4 with NaOH.
Statistical analysis

Drug concentrations and method of application

GABA was prepared as a 10 mM stock solution in HEPES solution. Clonazepam (CNZ) and zolpidem were first dissolved in dimethyl sulphoxide (DMSO) at 100 and 10 mM, respectively, and then diluted to the final concentration in HEPES medium. The maximum concentration of DMSO used in cellular perfusions was <0.001%, and this DMSO concentration has been shown not to alter GABA responses (Oh et al. 1995). The applied drug concentrations were as follows: GABA (Sigma, St. Louis, MO), 3–1,000 μM; CNZ (Sigma), 100 mM; zolpidem (RBI, Natick, MA), 100 nM; and zing (Sigma), 0.1–300 μM. Solution changes were accomplished with the use of a 13-barrel modified ‘‘sewer pipe’’ perfusion technique (Gibbs et al. 1996a,c), in which several solutions flowed out of parallel teflon tubes (0.2 mm ID) in a laminar fashion. Rapid (40–200 ms) and complete solution changes at a constant flow rate were then effected by moving the tube assembly laterally in relation to the neuron under study. No cross contamination was ever evident. After the seal was broken to institute whole cell recording mode, and ~2–5 min were allowed to pass to establish stable leak currents (0 to ~200 pA), GABA was applied for 4–6 s and washed out with control external solution for 30–40 s. The cell was pretreated with test drugs without GABA for 50–60 s and then test solutions were applied together with GABA. Drug effects with CNZ, zolpidem, and zing were expressed as percentage change from integrating the current response to a small depolarizing step. Current density was calculated on the basis of the maximal conductance increase (Fig. 1). This response was due to activation of GABA\textsubscript{A}Rs because 1) the intracellular electrode solution contained no potassium, which would preclude outward movement of potassium ions; 2) this response was blocked in a concentration-dependent manner by the GABA\textsubscript{A}R antagonists bicuculline (10–50 μM) and picrotoxin (1–100 μM) (Gibbs et al. 1996a, 1997; Oh et al. 1995); and 3) the reversal of the GABA-activated conductance was near to that predicted for a GABA\textsubscript{A}-evoked chloride conductance (Gibbs et al. 1996c; Oh et al. 1995). The theoretical reversal potential of GABA-evoked responses ($E_{\text{GABA}}$) was calculated to be $-70$ mV with the use of the Goldman-Hodgkin-Katz equation for a chloride conductance (Goldman 1943; Hodgkin and Katz 1949), assuming a phospho-to-chloride permeability ratio of 0.025 and an activity coefficient of 0.75 for the 166 mM external chloride solution (Bormann et al. 1987). The $E_{\text{GABA}}$ in control DG neurons was $-67.3 \pm 3.3$ (SE) mV ($n = 3$; all values are means ± SE here and below), compared with $-63.9 \pm 1.4$ mV ($n = 7$) in pilocarpine DG neurons. These reversal potentials were not statistically different ($P > 0.05$, t-test).

To evaluate the possible occurrence of alterations in functional GABA\textsubscript{A}R current density induced by epileptogenesis, GABA concentration-response curves were plotted for responses of DG cells to GABA applied in concentrations ranging from 3 to 1,000 μM. When plotted in a log GABA
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**A**
Control

Pilocarpine

GABA responses in dentate granule (DG) neurons isolated from control and epileptic (pilocarpine-treated) animals. A: traces of responses to varying concentrations of GABA in control and epileptic DG neurons illustrating the increase in the amplitude of GABA-evoked currents in pilocarpine DG neurons relative to controls. B: GABA concentration-response curves of control ($n = 10$) and pilocarpine ($n = 8$) DG neurons. Dentate cells of similar capacitance (range 5.8–13.2 pF) were chosen to normalize for potential membrane area differences. The concentration-response curve for the pilocarpine DG neurons showed a 109 and 89% enhanced efficacy of GABA at 100 μM and 1 mM, respectively, with no difference in the potency of GABA in between groups.

concentration vs. amplitude of GABA current graph, the GABA-evoked current was always found to increase in a sigmoidal manner as greater concentrations of GABA were applied to both control and epileptic DG cells (Fig. 1). The effect of application of increasing concentrations of GABA plateaued at 1 mM, as has been reported previously in acutely isolated rat (Gibbs et al. 1996a; Oh et al. 1995) and human cortical neurons (Gibbs et al. 1996c). GABA concentration-response curves were best fitted employing a nonlinear least-squares method assuming a monophasic sigmoidal GABA concentration-response relationship with the use of the equation

$$I = I_{max} C^n/(C^n + EC_{50}^n)$$

where $C$ is the GABA concentration, $I$ is the current elicited by a given GABA concentration, $I_{max}$ is the maximal GABA current, $EC_{50}$ is the GABA concentration eliciting half-maximal current, and $H$ is the Hill coefficient. Whole cell GABA-evoked currents of neurons with similar capacitance (6–13 pF) were analyzed and compared to try to control for the effects of any cellular size differences between treatment groups. A significant increase in the overall efficacy of GABA in evoking postsynaptic GABAAR responses was observed in DG neurons isolated from epileptic animals relative to controls (Fig. 1). The amplitude of GABA$_A$ responses to 100 μM and 1 mM GABA were significantly increased by 109.2 and 88.8%, respectively, in DG pilocarpine neurons relative to controls ($P < 0.003$ and $P < 0.004$, respectively, $t$-test). This increase in GABA efficacy associated with epileptogenesis in DG neurons isolated from spontaneously epileptic pilocarpine-treated animals was not accompanied by any significant change in the apparent potency of GABA in activating GABA currents. The best-fit $EC_{50}$ for the GABA concentration-response relationship were $39.2 \pm 4.1$ μM and $33.2 \pm 2.1$ μM.
32.7 ± 4.0 μM in control and epileptic DG neurons, respectively (Fig. 3). These EC_{50} means were not significantly different (P = 0.46, not significant, F test). To more thoroughly characterize the GABA efficacy differences described above, GABA_{AR} current densities (the maximal GABA current normalized to cellular capacitance) were quantified in control and epileptic DG neurons after application of a saturating concentration (1 mM) of GABA (see METHODS for specific details of this calculation). The capacitance-normalized GABA_{AR} current density significantly increased in epileptic DG neurons (4.22 ± 0.48 pA/μm^2; n = 14) in comparison with control neurons (2.37 ± 0.28 pA/μm^2; n = 16). This reflects a 78% higher GABA current density in epileptic compared with control DG neurons (P < 0.002, t-test).

**Epilepsy-associated alterations in postsynaptic GABA responses in CA1 neurons**

As was seen in control and epileptic DG neurons (described above), E_{GABA} showed no significant differences in CA1 neurons of epileptic animals compared with controls. E_{GABA} was −66.0 ± 2.0 mV in control CA1 neurons (n = 5) and −62.4 ± 4.3 mV in pilocarpine CA1 neurons (n = 4), values that were not statistically different (P > 0.05, t-test). To quantify possible alterations in functional GABA_{AR} current density in CA1 neurons induced by epileptogenesis, GABA concentration-response curves were plotted for data obtained in response to GABA application in concentrations ranging from 3 to 1000 μM in a manner identical to that described for DG cells (above). GABA concentration-response curves were fitted as described above (Fig. 2). Whole cell GABA-evoked currents in neurons with similar capacitance were analyzed and compared to normalize for possible cell size differences between CA1 groups. Unlike in DG neurons, where an increased efficacy of GABA was evident in epileptic neurons (Fig. 1), in CA1 neurons a significant decrease in the overall efficacy of GABA in evoking postsynaptic GABA_{AR} responses was observed in neurons isolated from epileptic animals relative to controls (Fig. 2). GABA current amplitudes elicited by application of 100 μM and 1 mM GABA were significantly reduced by 43.0 and 45.4%, respectively (P < 0.02 and P < 0.01, respectively, t-test). To more accurately assess this GABA efficacy decrease in epileptic CA1 neurons relative to controls, GABA current densities (the maximal GABA current normalized to cellular capacitance) were quantified in control and epileptic DG neurons after application of a saturating concentration (1 mM) of GABA (as described above and in METHODS). Capacitance-normalized GABA current density decreased from 4.74 ± 0.92 pA/μm^2 (n = 18) to 2.32 ± 0.36 pA/μm^2 (n = 13) in control and epileptic CA1 neurons, respectively, a statistically significant 51% decrease (P < 0.05, t-test). The potency or EC_{50} of GABA_{AR} responses varied significantly between control and epileptic neurons (Fig. 3). Control CA1 neurons had an EC_{50} of 35.8 ± 2 μM, and epileptic neurons had an EC_{50} of 24.5 ± 2.3 μM, in a population of cells with similar capacitances ranging between 12.5 and 20 pF (Fig. 3). These EC_{50} values were significantly different (P < 0.03, F test).

**Epilepsy-associated alterations in zinc blockade of GABA-evoked currents in DG neurons**

DG neurons were voltage clamped at −24 mV, and 10 μM GABA was applied alone and concurrently with varying concentrations of zinc to examine zinc blockade of GABA-evoked currents. This concentration of GABA was on the rising phase of the concentration-response curve for DG neurons and produced minimal desensitization (Fig. 1). Application of zinc in concentrations of 0.1–300 μM resulted in a concentration-dependent, sigmoidally increasing blockade of GABA-evoked currents in both control and epileptic DG neurons (Fig. 4). The data could be best fitted assuming a sigmoidal concentration-response relationship with the use of the equation

\[%\text{Inhibition} = \frac{M_1 C^{nH}}{(C^{nH} + IC_{50}^{nH})}\]

where \(M_1\) is the maximal zinc effect, \(C\) is the concentration of zinc, \(n\) is the Hill coefficient, and \(IC_{50}\) is the zinc concentration at which half-maximal effect was seen. The actual maximal level of GABA blockade inducible by zinc could not be determined because zinc concentrations >300 μM were neurotoxic to acutely isolated DG and CA1 neurons.

Zinc was found to block GABA-evoked currents to a much greater extent in epileptic DG neurons than in controls (Fig. 4). At 100 and 300 μM, zinc blocked GABA-evoked currents by 32.2 ± 2.9% and 31.3 ± 3.8% in control DG neurons, and by 76.3 ± 1.5% and 90.8 ± 0.6% in epileptic DG neurons, respectively. This corresponds to an epilepsy-associated statistically significant 136 and 190% increase in zinc block of DG GABA currents at 100 and 300 μM (P < 0.0001 for both sets of means, t-test).

**Timm’s staining in control and epileptic hippocampi**

One frequent pathological correlate of TLE is the presence of aberrantly located mossy fiber terminals, which

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FIG. 2. GABA$_A$ responses in CA1 neurons isolated from control and epileptic (pilocarpine-treated) animals. A: traces of responses to varying concentrations of GABA in control and epileptic CA1 neurons illustrating the decrease in the amplitude of GABA-evoked currents in pilocarpine neurons relative to controls. B: GABA concentration-response curves of control ($n = 14$) and pilocarpine ($n = 8$) CA1 neurons. CA1 neurons of similar capacitance (range 12.8 ± 19.4 pF) were chosen to normalize for potential membrane area differences between groups. Note that the concentration-response curve for the pilocarpine neurons showed a 43 and 45% decrease in the efficacy of GABA at 100 \( \mu \)M and 1 mM, respectively.

often sprout back into the molecular layer of the dentate gyrus in brains of epileptic patients or animals, in addition to innervating their normal targets in the hilus and CA3 (Babb et al. 1991; Mello et al. 1993; Sutula et al. 1989; Tauck and Nadler 1985; reviewed in McNamara 1994). This aberrant sprouting can be visualized, because the mossy fiber terminals contain high concentrations of zinc, a heavy metal that can be selectively stained for with the use of Timm’s stain (Haug 1973). Transverse slices of rat brain prepared from most of the animals immediately dorsal and adjacent to the sections employed in the acute isolation experiments described above were stained with the use of the Timm’s method to look for the presence and severity of epilepsy-associated aberrant zinc-containing terminals in the dentate supragranular layer (Fig. 6). Figure 6 shows photomicrographs of three sections from three different animals stained for the presence of zinc-containing synaptic terminals. No mossy fiber sprouting was present in the control section (Fig. 6, top), and significant sprouting was apparent in the pilocarpine-treated sections, with Fig. 6, middle, corresponding to a Tauck-Nadler score of 2 and Fig. 6, bottom, to a score of 3 (see METHODS for details) (Tauck and Nadler 1985). With the use of this scoring system, pilocarpine animals exhibited significant sprouting of mossy fibers into the molecular layer of the dentate gyrus (mean Timm’s score of 1.7 ± 0.2, $n = 11$) and controls showed little or no sprouting (mean score of 0.1 ± 0.1, $n = 14$) ($P < 0.0001$, Mann-Whitney rank sum test). This mean level of staining in the pilocarpine animals in the present study corresponds to patches of heavy molecular layer staining in the dentate gyrus, interspersed with regions of sparser staining, or a continuous band of intermediate staining in the molecular layer (e.g., Fig. 6, middle) (Tauck and Nadler 1985).
FIG. 3. Normalized GABA concentration response curves of control and epileptic DG and CA1 neurons. A: GABA concentration-response curves of control (n = 10) and epileptic (n = 14) DG neurons with similar capacitances, normalized to the GABA response evoked by 1 mM GABA (a saturating response) to better illustrate potential differences in the potency of GABA between groups. Note that there was no difference in the EC$_{50}$ between the control and pilocarpine DG neurons. Control and pilocarpine DG neurons had best-fit EC$_{50}$s of 39.2 ± 4 μM and 32.7 ± 4 μM, respectively (not significantly different, P > 0.05, F test). B: GABA concentration-response curve in control (n = 14) and pilocarpine (n = 8) CA1 neurons with similar capacitances (range 12.5 ± 20 pF), normalized as in A. Note the leftward shift or increase in potency of GABA in the epileptic CA1 neurons in relation to controls, corresponding to best-fit EC$_{50}$s of 35.8 ± 2 μM and 24.5 ± 2 μM in control and pilocarpine CA1 neurons, respectively (significantly different, P < 0.03, F test).

Epilepsy-associated alterations in benzodiazepine augmentation of GABA-evoked currents in DG neurons

DG neurons were voltage clamped at −24 mV, and 10 μM GABA was applied alone and concurrently with 100 nM CNZ, a commonly prescribed anticonvulsant for the treatment of generalized and partial complex seizures (Dreifuss et al. 1975), to examine CNZ augmentation of GABA$_{A}$-evoked chloride currents. CNZ (100 nM), which has been previously shown to produce maximal augmentation of the GABA currents in most neurons under the present recording conditions (Gibbs et al. 1996a; Oh et al. 1995), was employed to examine possible epilepsy-associated alterations in benzodiazepine modulatory effects on DG neurons. CNZ (100 nM) augmented the GABA (10 μM) response by 41.0 ± 10.8% (n = 11) in control DG neurons and by 87.8 ± 10.8% (n = 20) in epileptic DG neurons, a statistically significant 114% increase in CNZ-evoked augmentation (P < 0.01, t-test) (Fig. 7).

CNZ is a broad-spectrum benzodiazepine, which acts at both benzodiazepine, (BZ$_1$) and BZ$_2$ receptors (reviewed in Macdonald and Olsen 1994). To more completely characterize epilepsy-associated alterations in the function of GABA$_{A}$Rs, the effects of zolpidem (100 nM) were also examined and compared between control and epileptic DG neurons. Zolpidem is more specific agonist for BZ$_1$ receptors...
FIG. 4. Traces and concentration inhibition curves illustrating zinc blockade of GABA-evoked currents in control and pilocarpine DG neurons. A: traces illustrating the blockade by zinc (0.1–300 μM) of currents evoked by GABA (10 μM). Note that increasing concentrations of zinc produced an increase in the levels of block of the GABA-evoked current in both control and epileptic DG neurons; however, the level of the GABA blockade by zinc was significantly greater in pilocarpine neurons than in controls. B: plots of the zinc concentration GABA blockade curves in control (n = 18) and pilocarpine (n = 12) DG neurons. At 100 and 300 μM, zinc blocked GABA-evoked currents by 32.2 ± 2.9% and 31.3 ± 3.8% in control DG neurons, and by 76.3 ± 1.5% and 90.8 ± 0.6% in epileptic DG neurons, respectively, corresponding to a significant 136 and 190% increase in zinc block of DG GABA currents (both sets of means are significantly different, P < 0.0001, t-test). Note there was no significant decrease in the potency of zinc in control and pilocarpine DG neurons, with best-fit zinc concentrations at which half-maximal effect was seen (IC50 s) of 27.5 and 44 μM, respectively (not significantly different, P > 0.05, F test). Best-fit maximal efficacy (M) of zinc was 37.6 and 100%, whereas Hill coefficients (H) were 1.0 and 0.6 for control and epileptic DG neurons, respectively.

DISCUSSION

Epilepsy-associated alterations in benzodiazepine augmentation of GABA-evoked currents in CA1

CNZ (100 nM) augmentation of CA1 neuron GABA currents was also studied, with the use of techniques identical to those employed in DG neurons (described above). In contrast to the epilepsy-associated enhanced in CNZ modulation of GABA-evoked currents evident in DG neurons, CNZ (100 nM) was much lower in efficacy in augmenting epileptic CA1 neuron GABA currents compared with controls. CNZ (100 nM) augmented GABA-evoked currents by 115.4 ± 9.3% in control CA1 neurons (n = 11), and by 22.5 ± 6.1% in epileptic neurons (n = 7) (Fig. 9), a statistically significant 81% decrease in CNZ efficacy (P < 0.001, t-test).

Significant alterations in GABA_A_R function were evident in epileptic hippocampal neurons relative to controls. In epileptic DG neurons, GABA_A,R current density increased 78% (Fig. 1), zinc blockade of GABA responses increased 190% (Fig. 4), and GABA_A,R augmentation by 100 nM CNZ increased 114% (Fig. 7), whereas augmentation by the BZ_2 agonist zolpidem (100 nM) decreased 66% relative to con-
FIG. 5. Traces and concentration inhibition curves illustrating zinc blockade of GABA-evoked currents in CA1 neurons isolated from control and epileptic (pilocarpine-treated) rats. A: traces illustrating the blockade by zinc (0.1–300 μM) of currents evoked by GABA (10 μM). Increasing concentrations of zinc produced a similar increase in the levels of block of the GABA-evoked current in both control and epileptic neurons.

B: zinc concentration vs. GABA blockade curves for control (n = 20) and epileptic (n = 12) CA1 neurons. Zinc, at concentrations of 100 and 300 μM, blocked 61.1 ± 1.8% and 77.0 ± 1.5% of the GABA current in control neurons, and 52.4 ± 3.4% and 68.8 ± 2.5% in pilocarpine CA1 neurons, corresponding to a modest 17% and 12% decrease in pilocarpine cells relative to controls. Note there was no significant decrease in potency of zinc in blocking GABA receptors in CA1 neurons isolated from epileptic animals, with best-fit zinc IC50s of 28.3 ± 5.8 μM and 62.7 ± 24.7 μM (not significantly different, P > 0.05, F test). Best-fit maximal efficacy (M) of zinc was 90.1 and 98.3%, whereas Hill coefficients (H) were 0.7 and 0.5 for control and epileptic CA1 neurons, respectively.
FIG. 6. Timm’s stain of 25-μm-thick brain sections containing the ventral hippocampus in control and pilocarpine specimens. Low-power (A, ×4) and higher-power (B, ×10) views of ventral hippocampal sections from control (top) and 2 different pilocarpine (middle and bottom) rats. Note the pronounced aberrant mossy fiber “sprouting” in the supragranular layer in the dentate gyrus of the pilocarpine hippocampal sections, with no supragranular staining apparent in the control section. Middle: section corresponding to a Tauck-Nadler Timm score of 2. Bottom: section corresponding to a Timm score of 3. Calibration bar: 250 μm (A), 100 μm (B).

particularly in mIPSC studies involving random activity in hundreds of synapses, makes identification and quantification of smaller amplitude changes difficult. A 30% block of GABA_A may not have been apparent in these studies. In addition, the nature of the zinc blockade, which has been described as intermediate between competitive and noncompetitive (Celentano et al. 1991), makes it difficult to predict the exact effect the 30% block by 100–300 μM zinc of the current evoked by GABA (10 μM), described pharmacologically (Fig. 4), would have on a synapse, where millimolar GABA concentrations are achieved in the synaptic cleft.

Enhanced zinc sensitivity of epileptic DG GABA_ARs may be functionally important. Zinc localizes to mossy fiber terminals, and can be released in high concentrations (>100 μM) (Assaf and Chung 1984). Normally, mossy fibers do not “autoinnervate” DG neurons. With kindling, there are modest dentate gyrus circuit rearrangements, with fairly sparse levels of “sprouted” mossy fiber terminals in the supragranular layer (Cavazos et al. 1991; Sutula et al. 1989). Although GABA_A Rs zinc block is enhanced in kindled dentate gyrus (Buhl et al. 1996), there is a low density of sprouted zinc-containing terminals. This contrasts with the pilocarpine model, in which robust mossy fiber sprouting is evident (Mello et al. 1993; Okazaki et al. 1995) (Fig. 6). Thus, in pilocarpine animals, enhanced zinc block of DG GABA_A Rs and robust autoinnervation by zinc-containing
mossy fiber terminals coexist. Given that zinc can diffuse from its release site and interact with neighboring inhibitory receptors, the combined result of sprouting and enhanced zinc sensitivity could be dynamic disinhibition occurring during seizure discharges, or, as it was termed by Buhl et al. (1996), “a zinc-induced collapse of augmented inhibition” within the dentate gyrus.

Decreased functional GABA_{A}R current density (Fig. 2), enhanced potency of GABA (Fig. 3), and decreased benzodiazepine modulation of GABA_{A}R_{S} (Fig. 9) described in epileptic CA1 neurons in the present study constitute a “fingerprint” delineating a series of GABA_{A}R changes also seen in other studies in epileptic hippocampal and neocortical pyramidal neurons. In biochemical studies in kindled animals, [3H] muscimol binding and Cl\(^{-}\) influx decreased in CA1 compared with controls (Titulaer et al. 1994, 1995), consistent with the decreased GABA_{A}R current density in CA1 described in pilocarpine TLE rats (Fig. 2). In hippocampal cultures in which recurrent spontaneous seizure discharges can be induced by a one-time exposure to low-Mg\(^{2+}\) medium (Sombati and DeLorenzo 1995), this symptomatic fingerprint of GABA_{A}R alterations was evident accompanying develop-
ment of spontaneous epileptiform activity, and persisted for
the life of the culture (Gibbs et al. 1997). GABA$_A$R responses
in human neocortical pyramidal neurons isolated from an epi-
leptic focus exhibited similar decreased functional GABA$_A$R
density, increased GABA potency, and decreased benzodiazepine
efficacy (Gibbs et al. 1996b) when compared with nonfo-
cal pyramidal neuron GABA$_A$R responses (Gibbs et al.
1996c). This consistent pattern of epilepsy-associated GA-
BA$_A$R alterations in pyramidal neurons in both epilepsy model
systems and in humans suggests that these modifications may
be important in seizure susceptibility, irrespective of the
mechanisms eliciting this disorder.

**FIG. 8.** Zolpidem (ZOL) modulation of GABA-evoked currents in DG neurons iso-
olated from control and epileptic (pilocar-
pine-treated) animals. A: traces illustrating
the modulation by ZOL (100 nM) of the
current evoked by GABA (10 $\mu$M) in con-
trol and epileptic DG neurons. Note the de-
creased efficacy of ZOL in the pilocarpine
DG neurons relative to controls. B: histo-
gram illustrating the decreased efficacy of
ZOL in augmenting GABA responses in
pilocarpine DG neurons ($n = 10$) relative
to controls ($n = 7$). ZOL (100 nM) aug-
mented the GABA response by 158.1 ±
26.1% and 54.2 ± 7.3% in control and pilo-
carpine neurons, respectively. This was a
66% decrease in ZOL efficacy accompa-
nying epileptogenesis. Asterisk: signifi-
cantly different from control mean, $P <$
0.02, $t$-test.
Cloning/expresson studies detailing functional properties of GABA<sub>A</sub>Rs associated with specific subunit assemblies provide testable hypotheses concerning possible epilepsy-associated GABA<sub>A</sub>R structural rearrangements underlying regionally distinct alterations in ensemble cellular inhibitory function evident in this study. GABA<sub>A</sub>Rs are heteromers composed of several distinct protein classes, encoded by α-, β-, γ-, δ-, and ρ-families of mRNA subunits. The γ-subunit, when coexpressed with α- and β-subunits, confers benzodiazepine sensitivity to GABA<sub>A</sub>Rs, and influences zinc sensitivity (Draguhn et al. 1990; Saxena and Macdonald 1994; reviewed in Macdonald and Olsen 1994). GABA<sub>A</sub>Rs consisting of α- and β-subunits were potently blocked by zinc, whereas γ-subunit addition resulted in GABA<sub>A</sub>Rs that were insensitive to zinc (Draguhn et al. 1990). Replacement of the γ- with a δ-subunit in α,β-containing GABA<sub>A</sub>Rs conferred zinc sensitivity (Saxena and Macdonald 1994). However, hippocampal neuronal (γ-containing) GABA<sub>A</sub>Rs are zinc sensitive (Legendre and Westbrook 1990; Westbrook and Mayer 1987), and significantly reduced zinc sensitivity.

FIG. 9. CNZ modulation of GABA-evoked current in CA1 neurons isolated from control and epileptic (pilocarpine-treated) animals. A: traces illustrating the modulation by CNZ (100 nM) of the current evoked by GABA (10 μM) in control and pilocarpine CA1 neurons. Note the decreased efficacy of CNZ in the pilocarpine neurons relative to controls. B: histogram illustrating the decreased efficacy of CNZ in augmenting GABA responses in pilocarpine CA1 neurons (n = 7) relative to controls (n = 11). CNZ (100 nM) augmented the GABA response by 115.4 ± 9.3% and 22.5 ± 6.1% in control and pilocarpine CA1 neurons, respectively, corresponding to an 81% decrease in CNZ efficacy relative to controls. Asterisk: significantly different from control mean, P < 0.001, t-test.
TABLE 1. Summary of epilepsy-associated alterations in function of hippocampal neuron GABA<sub>A</sub> receptors

<table>
<thead>
<tr>
<th>Effect</th>
<th>Dentate</th>
<th>CA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA efficacy</td>
<td>↑↑</td>
<td>↓</td>
</tr>
<tr>
<td>GABA potency</td>
<td>↔</td>
<td>↑</td>
</tr>
<tr>
<td>Zinc blockade</td>
<td>↑↑</td>
<td>↔</td>
</tr>
<tr>
<td>CNZ augmentation</td>
<td>↑↑</td>
<td>↓</td>
</tr>
<tr>
<td>ZOL augmentation</td>
<td>↑↑</td>
<td>ND</td>
</tr>
</tbody>
</table>

↑↑: >50% Elevation in response amplitude or potency. ↑: 25–50% Elevation in response amplitude or potency. ↓: >50% Decrease in response amplitude or potency. ↔: 25–50% decrease in response amplitude or potency. ।: no effect. ND: not determined. GABA, γ-aminobutyric acid; CNZ, clonazepam; ZOL, zolpidem.

is evident in α<sub>1</sub>- compared with α<sub>2</sub>- and α<sub>3</sub>-containing GABA<sub>A</sub>Rs coexpressed with β- and γ-subunits (White and Gurley 1995). Epileptic DG GABA<sub>A</sub>R responses were 190% more sensitive to zinc blockade (Fig. 4). Furthermore, CNZ (100 nM) modulation of GABA<sub>A</sub>Rs was enhanced in epileptic DG neurons, whereas a BZ<sub>1</sub>-selective agonist was less effective (Figs. 7 and 8). This altered GABA<sub>A</sub>R pharmacology in DG neurons accompanying epileptogenesis could possibly be explained by an α-subunit switch in existing receptors, or incorporation of a new population of GABA<sub>A</sub>Rs containing α<sub>2</sub> or α<sub>3</sub> subunits, and exhibiting higher levels of BZ sensitivity. Epilepsy-associated enhanced incorporation of α<sub>3</sub> subunits into GABA<sub>A</sub>Rs in DG (Kamphius et al. 1995), associated with overall decreased contribution of α<sub>1</sub> and α<sub>2</sub> subunits due to simple stoichiometry, would confer increased zinc sensitivity (White and Gurley 1995) and decreased efficacy of zolpidem relative to CNZ in augmenting GABA responses, resulting in a shift from BZ<sub>1</sub> to BZ<sub>2</sub> GABA<sub>A</sub>R pharmacology of these neurons. Epilepsy-associated enhanced CNZ augmentation in DG neurons could result from enhanced expression of the γ<sub>2</sub> subunit (Kamphius et al. 1995), incorporation of new α<sub>2</sub>- or α<sub>3</sub>-containing receptors, or an α-subunit switch, because both α- and γ-subunits determine benzodiazepine modulation of GABA<sub>A</sub>Rs (reviewed in Macdonald and Olsen 1994). Epilepsy-associated decreased benzodiazepine sensitivity (Fig. 9) and enhanced GABA potency (Fig. 3) evident in CA1 neurons could be explained parsimoniously by reduced γ<sub>2</sub> subunit expression in the residual receptors. GABA more potently activates GABA<sub>A</sub>Rs lacking a γ-subunit, and these receptors would not be modulated by benzodiazepines.

Table 1 summarizes the findings of the present study, illustrating the overall effects of induction of an epileptic state on functional GABA receptor properties in hippocampal DG and CA1 neurons. What is most effectively illustrated are the contrasting epilepsy-associated changes evident in these two cell types. GABA efficacy was significantly elevated in epileptic DG neurons, and reduced in epileptic CA1 neurons, with little change in GABA potency evident in DG and a significantly enhanced potency of GABA evident in CA1. Zinc blockade of GABA currents was significantly enhanced in epileptic DG neurons, and unaffected in epileptic CA1 neurons, whereas CNZ augmentation of GABA responses was enhanced in DG neurons and reduced in CA1 neurons after development of an epileptic condition. These novel differential alterations in functional postsynaptic GABA responses in these distinct populations of hippocampal neurons could translate into significant and distinct alterations in circuit properties accompanying epileptogenesis in these areas of the hippocampus.

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