Long-lasting reduction of inhibitory function and γ -aminobutyric acid type A receptor subunit mRNA expression in a model of temporal lobe epilepsy

(gene expression)

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ABSTRACT This study evaluated hippocampal inhibitory function and the level of expression of γ -aminobutyric acid type A (GABA_A) receptor mRNA in an *in vivo* model of epilepsy. Chronic recurrent limbic seizures were induced in rats using injections of pilocarpine. Electrophysiological studies performed on hippocampal slices prepared from control and epileptic animals 1 to 2 months after pilocarpine injections demonstrated a significant hyperexcitability in the epileptic animals. Reduced levels of mRNA expression for the $\alpha 2$ and $\alpha 5$ subunits of the GABA_A receptors were evident in the CA1, CA2, and CA3 regions of the hippocampus of epileptic animals. No decrease in mRNA encoding $\alpha 1$, $\beta 2$, or $\gamma 2$ GABA_A receptor subunits was observed. In addition, no change in the mRNA levels of α CaM kinase II was seen. Selective decreases in mRNA expression did not correlate with neuronal cell loss. The results indicate that selective, long-lasting reduction of GABA_A subunit mRNA expression and increased excitability, possibly reflecting loss of GABAergic inhibition, occur in an *in vivo* model of partial complex epilepsy.

γ -Aminobutyric acid (GABA) is the major inhibitory neurotransmitter system in mammalian brain (1) and has been implicated in playing an important role in the induction and maintenance of epileptogenesis in several experimental models (2–6). In brain tissue from epileptic patients, decreased levels of GABA receptor-binding and of GABA-synthesizing enzymes have been documented (3). Furthermore, reduced benzodiazepine receptor binding in patients with temporal lobe epilepsy has been demonstrated *in vivo* by positron-emission tomography (7). In animal models of epilepsy, changes in the level of GABA type A (GABA_A) receptor binding have also been observed in specific hippocampal regions in kindled rats (8, 9). Studies from our laboratories have shown a decrease in GABA_A receptor binding in rat brain membranes (10) and a reduction in the physiological response to GABA in acutely isolated CA1 hippocampal neurons (11) after the induction of epileptogenesis and persistent seizure discharge by pilocarpine injection. Thus, a considerable body of evidence indicates that decreased GABA receptor function and/or expression may play an important role in the induction and maintenance of epilepsy.

Although GABA systems are altered in epilepsy, the specific molecular mechanisms mediating these changes have not been clearly elucidated (4, 6). Long-term changes in gene expression produced by the induction of epileptogenesis have been suggested to play an important role in the persistent hyperexcitability observed in chronic epilepsy (12, 13). Studies from our laboratory have demonstrated persistent changes in gene

expression for up to 6 weeks in the hippocampus of kindled rats (13). These results indicate that changes in gene expression may underlie the permanent alterations in neuronal excitability induced during epileptogenesis and suggest that decreased genetic expression of the GABA_A receptor subunits may underlie the reduced inhibition observed in several models of epilepsy. This study was initiated to examine the effect of the induction of epileptogenesis in an *in vivo* model of partial epilepsy on the expression of GABA_A receptor mRNA and inhibitory function within the hippocampus. The results demonstrate that persistent loss of inhibition and long-lasting reduction of select GABA_A subunit mRNA expression within the hippocampus accompany the development of chronic recurrent seizures in this *in vivo* model of epilepsy and suggest that regulation of the expression of GABA_A subunit gene expression may be an important mechanism mediating decreased inhibition and contributing to the induction of epileptogenesis.

MATERIALS AND METHODS

Adult female Sprague-Dawley rats, weighing 150 to 200 g, were housed for 1 to 2 weeks before experiments were performed. Experimental animals were injected with pilocarpine (350 mg/kg i.p.), preceded by 30 min with methyl scopolamine (1 mg/kg i.p.) to reduce peripheral pilocarpine effects or appropriate control vehicle solution using established procedures (14, 15). Within 20 to 40 min after the pilocarpine injection, essentially all of the animals developed status epilepticus (SE). SE was allowed to continue for 1 hr and then control and experimental animals were treated with diazepam (4 mg/kg i.p.). If clinical seizures returned, animals were given a second diazepam injection. Animals recovered from this initial treatment within 2 to 3 days and were observed for the next 2 months. Animals were monitored by video recording and by clinical observation to evaluate the development of seizure discharge. Seizures were scored on a scale from 1 to 5, as used for the scoring of kindled seizures as described by Racine (16). Over 60% of the animals were found to have recurrent partial and generalized seizures between 1 and 2 months after the initial pilocarpine injection. No seizures were observed in sham-injected control animals. A separate set of experimental and control animals had hippocampal depth electrodes implanted (13). Continuous electrophysiological and video monitoring (Telefactor Instruments, Philadelphia) were performed on these animals and demonstrated electrographic

Abbreviations: GABA, γ -aminobutyric acid; GABA_A, GABA type A; SE, status epilepticus; HEC, hippocampal-entorhinal cortex; EEG, electroencephalogram.

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seizure discharges in animals undergoing clinical seizures. Combined electrophysiological and video recording demonstrated a correlation between electrographic seizure discharge and clinical seizure activity. No electrographic or behavioral seizure activities were observed in the sham-treated animals. Animals were observed for 24 hr before they were killed. Only animals that had no seizure activity for 24 hr before they were killed were used for experimental studies.

Electrophysiological Studies. Slice preparation. Electrophysiological experiments were carried out on hippocampal-entorhinal cortical (HEC) slices from pilocarpine-treated rats 2 months after treatment as described (17, 18). Sham controls or pilocarpine-treated rats were decapitated after halothane anesthesia. The whole brain was removed and placed in cold, oxygenated sucrose-based artificial cerebrospinal fluid containing 200 mM sucrose, 3 mM KCl, 1.25 mM Na_2PO_4 , 26 mM NaHCO_3 , 10 mM glucose, 0.5–0.9 mM MgCl_2 , 2 mM CaCl_2 . After a 2-min incubation, each hemisphere of the brain was individually blocked and sectioned with a vibratome (Lancer 1000, St. Louis) along a 12° inclined transverse plane (19) and at 450 μm thickness. Sectioned HEC slices were further dissected manually with a scalpel to isolate the hippocampus with its adjoining entorhinal and temporal cortex. The HEC slices retained an intact circuit within the hippocampus in conjunction with adjoining cortical regions (17). After dissection, the HEC slices were incubated for 1 hr in warmed (32°C) and oxygenated (95% $\text{O}_2/5\%$ CO_2) artificial cerebrospinal fluid (ACSF) containing 130 mM NaCl, 3 mM KCl, 1.25 mM Na_2PO_4 , 26 mM NaHCO_3 , 10 mM glucose, 0.5 mM MgCl_2 , 2 mM CaCl_2 . Individual HEC slices were transferred following incubation into an interface chamber (Medical Systems, Greenvale, NY) and perfused with warmed (35°C) ACSF 0.5–1.0 ml per min for extracellular recording experiments.

Electrophysiological experimental protocol. Insulated tungsten electrodes (A-M Systems, Everett, WA) were placed in the hippocampal pyramidal cell body layer of area CA1. Amplified signals were digitized with a PCM device at 8 kHz per channel (NeuroData Instruments, New York), recorded onto videotape, and played back off line for analysis using PCLAMP software (Axon Instruments, Foster City, CA). Input-output curves were plotted out to evaluate the level of stimulation producing maximal population spike amplitude.

In Situ Hybridization. For *in situ* hybridization, coronal and sagittal sections were prepared from control and experimental animals as described previously (13). Rats were anesthetized with halothane and perfused with 300 mM sucrose (4°C). Following perfusion, the brain was rapidly removed and frozen in liquid nitrogen chilled isopentane. Sections of cortex were blocked in OCT compound, cryosectioned (10- μm thick sections), mounted on 1% gelatin-coated microscope slides, and stored at -70°C until used. When needed, sections were thawed and fixed in 4% paraformaldehyde phosphate-buffered saline (PBS) for 20 min and permeabilized in 70% ethanol for 1 hr. The sections were probed with oligonucleotides complementary to mRNA for $\alpha 1$, $\alpha 2$, $\alpha 5$, $\beta 2$, and $\gamma 2$, for GABA_A subunits (20, 21), and for α Cam kinase II (22). The sections were prehybridized at 42°C for 4 hr, followed by hybridization with ^{35}S -labeled probe for 20 hr at 42°C . Sections were then washed three times for 30 min each in $1\times$ standard saline citrate at 55°C . Sections were dehydrated, air-dried overnight, and exposed to Amersham hyper-film MP. Complementary oligonucleotide probes to the GABA_A $\alpha 1$, $\alpha 2$, $\alpha 5$, $\beta 2$, and $\gamma 2$ and α Cam kinase II subunits were 3'-end labeled with [α - ^{35}S]dCTP (terminaldeoxytransferase reaction) for 3 hr at 37°C . The specific activity of each probe was approximately 10^6 cpm per section. Autoradiographic images were analyzed by a Loates (Westminster, MD) image analysis system. Treatment of sections with RNase T1 before hybridization was used to evaluate the specificity of the hybridization signal. In addition, the specificity of each probe was verified in Northern

blot analysis of rat hippocampal mRNA using established procedures (23).

RESULTS

Electrographic and Clinical Seizure Activity. Representative hippocampal depth electrode recordings from a control and epileptic animal are presented in Fig. 1. Control animals and parts of the interictal electroencephalogram (EEG) tracings of epileptic animals manifested normal background activity (Fig. 1 *A* and *B*). Interictal spike discharges were seen intermittently in animals manifesting clinical seizure activity (Fig. 1*B*). The tracings shown in Fig. 1 *B* and *C* were obtained during a clinical seizure that was simultaneously recorded on video EEG. These recordings document that hippocampal electrographic seizure discharges occurred during clinical seizure activity. The seizure shown in Fig. 1 demonstrates the development of tonic and tonic-clonic seizure discharges.

Long-Lasting Loss of Inhibition. Extracellular stimulation-induced activation of the Schaeffer collaterals triggered normal excitatory postsynaptic potential/population spike responses in CA1 recordings from control HEC slices (Fig. 2). However, in HEC slices prepared from pilocarpine-treated animals, the identical stimulation paradigm triggered long bursts of multiple population spikes, indicating diminished inhibition or enhanced excitation in these animals, potentially contributing to increased susceptibility to support epileptic activity (Fig. 2*A*). Quantitation of the number of population spikes in both groups demonstrated a statistically significant 3.6-fold increase in the number of action potentials elicited by a single stimulus in epileptic animals, as determined using the Whitney–Mann rank sum test (Fig. 2*B*). Similar results were obtained from CA3 recordings.

FIG. 1. Hippocampal depth electrode EEG recordings from representative control (*A*) and epileptic (*B* and *C*) animals 2 months after vehicle or pilocarpine injections. (*A*) Control animals demonstrate normal background EEG rhythms. (*B*) Epileptic animals consistently manifested interictal spike discharges during EEG recordings; a typical seizure record is shown. Rapid spike discharges with frequencies between 10 and 20 Hz (region 1), followed by well-formed polyspike and slow wave discharges (region 2) were typically observed (*C*) and corresponded in time to the tonic and clonic phases of the clinical seizure activity that was observed on the video EEG.

FIG. 2. Field potential activity evoked by Schaeffer collateral stimulation in the CA1 region. (A) Schaeffer collateral stimulation (7 V, 100 μ sec) elicited a single population spike in control HEC slices ($n = 5$) and polyspiking in slices from pilocarpine-treated animals ($n = 6$). (B) Quantitation of single pulse-induced population spike responses in HEC slices from control and pilocarpine-treated animals. The data represent the mean $\pm$ SEM (*, $P \leq 0.03$).

Long-Lasting Reduction of Specific GABA Receptor Subunit mRNA Expression. Sections of brain from control and epileptic animals were analyzed by *in situ* hybridization techniques to evaluate the expression levels of GABA_A receptor, $\alpha 1$, $\alpha 2$, $\alpha 5$, $\beta 2$, and $\gamma 2$ subunit mRNA. The patterns of GABA_A receptor subunit mRNA expression are shown in Fig. 3. All of the GABA_A receptor subunits evaluated were expressed in the hippocampus and were distributed within the DG, CA1, CA2, and CA3 regions, as previously described by Poulter *et al.* (21). The pseudocolor autoradiographic images shown in Fig. 3 demonstrate qualitatively that mRNAs for GABA_A receptor subunits $\alpha 2$ and $\alpha 5$ were decreased in the CA1, CA2, and CA3 regions of the hippocampus in the epileptic rats compared with controls. The expression of the $\alpha 5$ subunit mRNA was slightly increased in the DG. No changes were seen in mRNAs encoding the $\alpha 1$, $\beta 2$, and $\gamma 2$ GABA receptor subunits.

To quantitate the changes in mRNA levels shown qualitatively in Fig. 3, we conducted a densitometric analysis of the *in situ* hybridization autoradiographs (13). A statistically significant reduction in GABA_A receptor $\alpha 2$ and $\alpha 5$ subunit mRNA levels was found in the epileptic animals in multiple experiments (Fig. 4). GABA_A receptor subunit $\alpha 5$ mRNA levels were decreased 25.4% in CA1-CA2 region and 20.1% in CA3 region in the seizure animals. GABA_A receptor subunit $\alpha 2$ mRNA expression was also significantly reduced by 14.3% in CA1 and CA2 regions and 11.1% in CA3 region. In the same animals that manifested reduced expression of mRNA for $\alpha 2$ and $\alpha 5$ subunits, no significant changes in the GABA_A recep-

FIG. 3. Representative *in situ* hybridization hippocampal patterns for $\alpha 1$, $\alpha 2$, $\alpha 5$, $\beta 2$, and $\gamma 2$, GABA_A receptor subunit expression from control and epileptic animals. Data are shown as pseudocolor images produced on a Loates quantitative densitometric system with the intensity scale shown below. Results shown are representative of eight separate experiments. The highest levels of GABA_A receptor subunit mRNA were observed in the neuronal cell body layers of the DG, CA1-2, and CA3 regions of the hippocampus.

tor $\alpha 1$, $\beta 2$, and $\gamma 2$ subunit mRNA levels were found (Fig. 3). A statistically significant but minor increase in $\alpha 5$ subunit mRNA expression (12.3%) was observed in DG. However, no changes in mRNAs for GABA_A receptor $\alpha 1$, $\alpha 2$, $\beta 2$, or $\gamma 2$ subunits were observed in the DG. To further evaluate the selectivity of this effect we examined message levels of α CaM kinase II; these levels were not significantly altered by the development of chronic seizure discharges in the same preparations that showed statistically significant changes in specific GABA_A receptor subunit mRNAs (data not shown).

To evaluate the specificity of the *in situ* hybridization signal, we treated brain sections with RNase T1 before the addition of complementary RNA probes. RNase T1 digestion resulted in the loss of the *in situ* hybridization signal. Because the cerebral cortex did not show statistically significant changes in mRNA levels for either GABA_A receptor subunits or CaM kinase II, we normalized the level of mRNA expression for each probe to that in the cortex to control for variability in

FIG. 4. Quantitation of GABA_A receptor subunit mRNA expression. *In situ* hybridization patterns shown qualitatively in Fig. 3 were quantitated using the Loates quantitative densitometric system for the GABA_A $\alpha 1$, $\alpha 2$, $\alpha 5$, $\beta 2$, and $\gamma 2$ subunits in the CA1-CA2 (A), CA3 (B), and DG (C) regions of the hippocampus. The data give the values for the epileptic animals expressed as a percentage of control animals ($n = 8$). The data are expressed as the mean $\pm$ SEM. Because mRNA levels for the GABA_A receptor subtypes in cortical tissue in control animals were not significantly changed from epileptic animals, we expressed the hippocampal mRNA levels relative to cortical mRNA levels to control for variability in hybridization. In addition, data are controlled for the mean number of neurons per unit area in each region for control and epileptic animals. *, $P \leq 0.01$; **, $P \leq 0.005$; ***, $P \leq 0.001$, as determined by Student's *t* test.

probe hybridization. Since a decrease in pyramidal neurons in CA1-CA2 and CA3 regions of the hippocampus has been observed in chronic pilocarpine treated animals (14, 24), we determined cell numbers per unit area in the DG, CA3, and CA1-CA2 regions in adjacent hippocampal sections to those used for *in situ* hybridization. No change in cell number was found in the DG, but a 15% decrease in CA3 and an 11% decrease in CA1-CA2 cell number was observed in epileptic compared with control animals in the experiment shown in Fig. 4. Thus, we normalized the *in situ* quantitation data to cell number so that the data shown in Fig. 4 was controlled for the loss of neurons seen in CA1-CA2 and CA3 regions.

DISCUSSION

These studies demonstrate that selective long-lasting changes in GABA_A hippocampal receptor subunit mRNA levels accompany a loss of inhibition in an *in vivo* model of partial epilepsy. Statistically significant decreases in the levels of $\alpha 2$ and $\alpha 5$ subunit mRNA were observed in the CA1-CA2 and CA3 regions of the hippocampus. Conversely, a statistically significant increase in the level of $\alpha 5$ GABA_A receptor subunit mRNA was observed in the DG. These changes in GABA_A receptor gene expression occurred in the same experimental animals that did not manifest changes in the levels of $\alpha 1$, $\beta 2$, and $\gamma 2$ subunit mRNA or of the α CaM kinase subunit. These

results demonstrate that loss of inhibition and decreased expression of specific GABA_A receptor subunit mRNA expression are associated with the development of recurrent seizures and suggest that alterations in the expression of specific GABA_A receptors during epileptogenesis may be an underlying molecular mechanism mediating some of the loss of inhibition seen in this animal model of epilepsy.

Sloviter (5, 6) and Bekenstein and Lothman (25) have provided convincing evidence for a dormant inhibitory interneuron hypothesis of epileptogenesis, indicating that in some forms of epileptogenesis, inhibitory neurons are present but are not recruited in the signal transmission process because of a deficiency in the stimulatory inputs into these inhibitory neurons. However, studies by Merlin and Wong (26) demonstrate that epileptogenesis in the hippocampal slice preparation can occur under conditions that exclude either the loss of stimulation onto the interneuron or the dormant cell interneuron hypothesis. Instead, their studies indicate that changes in the inhibitory synaptic junction and altered excitability of the interneuron may also contribute to epileptogenesis. In our previous studies of the acute pilocarpine model of temporal lobe epilepsy, both a decrease in GABA receptor binding (10) and a decrease in the effectiveness of GABA to hyperpolarize pyramidal cells isolated from hippocampal slices of treated animals (11) were associated with epileptogenesis. Loss of GABA_A inhibition has also been demonstrated in association with chronic epileptogenicity following SE (27). Changes in GABA_A receptor function and GABA inhibition have also been observed in kindled rat hippocampus (8, 9). Although all of these models implicate a decrement in inhibition, they differ in the manner in which inhibition is altered. Whether this difference reflects the regulatory level affected in each model, the manner in which seizures are induced (chemical versus electrical), or the degree of cell loss requires further investigation.

An important molecular mechanism that could account for reduced inhibition during epileptogenesis is a decrease in the expression of GABA_A receptor subunits. Studies in the kindling model of epilepsy have demonstrated that decreased muscarinic and benzodiazepine binding have been observed for as long as a month after the initial kindling treatment in the CA1 and CA2 regions of the hippocampus (8, 9, 28). Increased GABA receptor binding in the kindling model was also observed in the DG (8, 9), consistent with the increase in $\alpha 5$ mRNA in the DG observed in this paper. In addition, decreased GABA receptor binding and response to GABA have been observed in the pilocarpine model of temporal lobe epilepsy (10, 11). Mechanisms that alter the level of the GABA_A receptor subunits and influence GABA binding may be contributing to the loss of inhibition seen in induction of epileptogenesis. Thus, decreased gene expression of select GABA_A receptor subunits in the CA1-CA3 regions and an increase in DG may account for the enduring changes in GABA_A receptor function seen in several of these epilepsy models.

Changes in levels of mRNA encoding GABA_A receptor subunits have been observed following SE (29), electroconvulsive shock (30), and kindling-induced seizures (31). In addition, decreases in the mRNA levels of the GABA_A $\alpha 2$ receptor subunit were observed in association with the induction of epileptogenesis in an *in vitro* hippocampal slice model of epilepsy (32). Long-lasting depression of GABA_A receptor subunit mRNA levels has also been shown to occur with epileptogenesis and maintained for the life of neurons in culture in an *in vitro* hippocampal model of epilepsy (33, 34). In this same preparation, a selective knockdown of GABA_A receptor $\alpha 2$ subunit potentiated recurrent seizure discharges. Removal of the antisense oligonucleotides resulted in a gradual decline of seizure activity (35). Our findings indicate that reduction of GABA_A receptor mRNA expression occurs during epileptogenesis and is maintained for long periods of

time in several epilepsy preparations. Houser and colleagues (36) have independently reported findings that also indicate that GABA_A $\alpha 5$ mRNA subunit expression is chronically reduced in CA1 hippocampal neurons in the pilocarpine model of temporal lobe epilepsy. The association of decreased GABA_A receptor subunit mRNA and loss of inhibition in the pilocarpine model suggest that decreased receptor availability may contribute to loss of inhibition thereby contributing to the increased excitability evident in this model of epilepsy.

Although steady-state levels of mRNA generally govern the rate of protein synthesis and, hence, are indicative of the expression of any gene, it is premature to associate changes in selective GABA_A receptor gene expression with changes in its function. Further study is required to elucidate the functional roles and physiological consequences of down-regulation of select GABA_A receptor subunits. However, we have demonstrated previously that acutely isolated neurons from hippocampal slices prepared from pilocarpine treated animals manifested decreased GABA_A receptor binding and response to GABA stimulation compared with control animals (10). These studies directly evaluated the effect of GABA on isolated hippocampal neurons and strongly suggest that decreased receptor availability is present and may contribute to the loss of inhibition seen in this model. Our previous and present work raises the possibility that long term decreases in GABA_A subunit gene expression and decreased GABA_A receptor function during epileptogenesis may contribute to the induction and maintenance of seizure discharges in this model of partial complex epilepsy.

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