Physiological and Pharmacological Alterations in Postsynaptic GABA<sub>A</sub> Receptor Function in a Hippocampal Culture Model of Chronic Spontaneous Seizures

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Gibbs, John W., III, Sompong Sombati, Robert J. DeLorenzo, and Douglas A. Coulter. Physiological and pharmacological alterations in postsynaptic GABA<sub>A</sub> receptor function in a hippocampal culture model of chronic spontaneous seizures. J. Neurophysiol. 77: 2139–2152, 1997. Cultured rat hippocampal neurons previously exposed to a media containing no added Mg<sup>2+</sup> for 3 h begin to spontaneously trigger recurrent epileptiform discharges following return to normal medium, and this altered population epileptiform activity persisted for the life of the neurons in culture (2 wk). Neurons in “epileptic” cultures appeared similar in somatic and dendritic morphology and cellular density to controls, untreated cultures. In patch-clamp recordings from hippocampal pyramidal cells from “epileptic,” low Mg<sup>2+</sup>-pretreated hippocampal cultures, a rapid (within 2 h of treatment), permanent (lasting ≥8 days) and statistically significant 50–65% reduction in the current density of functional γ-aminobutyric acid-A (GABA<sub>A</sub>) receptors was evident when the GABA responses of these cells were compared with control neurons. Functional GABA receptor current density was calculated by determining the maximal response of a cell to GABA 1 mM application and normalizing this response to cellular capacitance. Despite the marked GABA efficacy differences noted above, the potency of GABA in activating chloride currents was not significantly different when the responses to control and “epileptic” pyramidal cells to multiple concentrations of GABA were compared. The EC<sub>50</sub> for GABA was 4.5 ± 0.2 (mean ± SE) for control neurons and 3.5 ± 0.4 μM, 5.2 ± 0.5 μM, 3.7 ± 0.3 μM, and 4.6 ± 0.3 μM for epileptic neurons 2 h, 2 days, 3 days, and 8 days after low Mg<sup>2+</sup>-pretreatment, respectively. Modulation of GABA responses by the benzodiazepine, clonazepam, was significantly reduced in epileptic neurons compared with controls. The kinetics determined clonazepam 100 nM GABA augmentation efficacy decreased from 44.1% in control neurons to 9.3% augmentation in neurons recorded from cultures 10 days posttreatment. The kinetics of GABA current block by the noncompetitive antagonist picrotoxin were determined in hippocampal cultured neurons, and an IC<sub>50</sub> of 14 μM determined. Bath application of picrotoxin at half of the IC<sub>50</sub> concentration (7 μM) induced epileptiform activity in control cultures and this activity appeared very similar to the epileptiform activity induced by prior low Mg<sup>2+</sup> treatment. This concentration of picrotoxin was determined experimentally to block 30% of the GABA<sub>A</sub>-mediated receptor responses in these cultures, and this level of block was sufficient to trigger spontaneous epileptiform activity. The 50% reduction of GABA responses induced as a permanent consequence of low Mg<sup>2+</sup> treatment therefore was determined to be sufficient in and of itself to induce the spontaneous epileptiform activity, which was also a consequence of this treatment.

INTRODUCTION

Human limbic epilepsy affects >1 million people in the United States alone, and thus represents a major health problem (Hauser and Hersdorf 1980). Limbic epilepsy is one of the most devastating forms of epilepsy in the adult population. About 60% of patients with intractable epilepsy (i.e., seizures that are not adequately controlled by medication) have partial seizures, and a significant proportion of these patients have seizures that originate in the limbic system (Mikati and Holmes 1993). Limbic epilepsy frequently is characterized electroencephalographically by complex partial seizures originating in the temporal lobe, with or without secondary generalization to adjacent and distant cortical areas (reviewed in Lothman et al. 1991). The limbic system, particularly the hippocampus, is uniquely vulnerable to develop the pathological synchronized electrical activity within populations of neurons that constitute epilepsy (Dichter and Ayala 1987; Lothman et al. 1991; McNamara 1994). One of the major long-term goals of research into the pathophysiology of limbic epilepsy is to understand the cellular and molecular mechanisms underlying this limbic vulnerability to develop seizure activity. Elucidating the mechanisms that cause limbic epilepsy may provide important insight into the treatment and prevention of this debilitating condition.

One research direction that has provided valuable information concerning mechanisms underlying limbic epilepsy has been the development of in vitro limbic model systems that can sustain epileptiform activity. For example, exposure of hippocampal/entorhinal cortical brain slices, thalamocortical slices, or hippocampal neurons in culture to an extracellular media containing no added Mg<sup>2+</sup> induces sustained spontaneous epileptiform activity in vitro during the treatment (Coulter and Lee 1993; Sombati and DeLorenzo 1995; Walther et al. 1986; Wilson et al. 1988; Zhang and Coulter 1996; Zhang et al. 1996a,b). Despite the ability to induce “acute” epileptiform activity through pharmacological and electrical manipulations, efforts directed toward induction of an epileptic state in normal medium have been largely unsuccessful in vitro. One such preparation has been de-
scribed recently, in which repetitive “kindling” stimuli trigger spontaneous recurrent electrographic seizure discharges in hippocampal-entorhinal cortical slices (Rafiq et al. 1993, 1995). Recently, two neuronal hippocampal culture models capable of supporting long-term, spontaneous self-sustained epileptiform activity in normal extracellular medium have been developed (Segal and Furshpan 1990; Sombati and DeLorenzo 1995). In the Sombati and DeLorenzo model, these spontaneous seizure-like events persisted for the life of the cultures (>2 wk) and were controlled effectively by clinically useful partial and generalized tonic-clonic anticonvulsant drugs, but not those effective in control of absence seizures (Sombati and DeLorenzo 1995). In this latter model, the epileptic state is created by kindling the cultures with a 3-h pretreatment in medium containing no added Mg$^{2+}$. This treatment elicits sustained seizure activity in the cultures and this activity then resolves after return to normal medium into recurrent electrographic seizure discharges activating large populations of neurons within the culture. This type of neuronal culture model capable of sustaining “chronic,” prolonged in vitro epileptiform activity may provide a powerful tool for the study of the cellular and molecular mechanisms potentially involved in limbic epileptogenesis.

In the present study, the potential role of diminished inhibitory efficacy within the “epileptic” hippocampal cultures in the generation of the epileptic state is examined using whole cell patch-clamp recording techniques.

METHODS

Hippocampal culture

Hippocampal neuronal cultures were prepared from hippocampal tissue isolated from 2-day-old Sprague-Dawley rats. Neurons were isolated, plated at a density of 2 × 10^5/cm^2 onto a confluent astroglial support layer, and maintained in a culture incubator for 3 wk at 37°C as has been described previously (Coutler et al. 1992; Sombati and DeLorenzo 1995; Sombati et al. 1991). Although cultures were of mixed cellular morphologies, only neurons with medium to large pyramidal shaped somas were recorded in the present study. Cultures were exposed to culture media containing no added Mg$^{2+}$ for 3 h and then returned to a regular culture media containing normal Mg$^{2+}$ levels, in which the cultures were maintained. Concentrations of Mg$^{2+}$ of >0.5 mM during the 3-h pretreatment were sufficient to block the subsequent development of epileptogenesis. Shorter exposures to low Mg$^{2+}$ (<1 h) did not consistently produce a permanent alteration in neuronal excitability. A preexposure to low Mg$^{2+}$ for 3 h and subsequent return to a regular culture media produced optimal epileptiform discharge activity. At the time of electrophysiological recording, the extracellular culture medium was replaced with a N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid (HEPES) solution composed of (in mM) 155 NaCl, 3 KCl, 1 MgCl$_2$, 3 CaCl$_2$, 0.0005 tetrodotoxin, and 10 HEPES-Na$^+$, with pH adjusted to 7.4 with NaOH.

Voltage-clamp recordings

Whole cell voltage-clamp recordings were conducted as previously described (Coutler et al. 1990; Gibbs et al. 1996a,c; Oh et al. 1995). The intracellular (pipette) solution contained (in mM) 100 Trizma phosphate ( dibasic ), 28 trizma base, 11 ethylene glycol bis-(-beta-aminopropyl ether)-N,N,N’ ,N’-tetraacetic acid, 2 MgCl$_2$, and 0.5 CaCl$_2$, with pH adjusted to 7.35 with NaOH. Whole cell patch-clamp recording techniques were conducted on a Nikon inverted microscope equipped with Hoffman modulation contrast optics. Electrodes (4–8 MΩ) were pulled on a Narishige PP-83 microelectrode two-stage puller with thin-walled borosilicate capillary glass (WPI, Sarasota, FL). The pipette solution also contained an intracellular ATP reconstitution system, consisting of 50 U/ml creatine phosphokinase, 22 mM phosphocreatine, and 4 mM Mg-ATP. The intracellular ATP maintenance solution was used to fill the shank of the electrode but was omitted from the solution that was used to back-fill the tip of the electrode. Recordings were amplified using an Axopatch 200A amplifier (Axon Instruments, Burlingame, CA) and filtered at 5 kHz with a 4-pole Bessel filter before digitization. All data were displayed on a chart recorder online (Gould, Model 2107, Cleveland, OH; frequency response DC-50 Hz) and stored on a VCR after digitization (44 kHz) with a PCM interface (Neurodata Instrument, New York). For subsequent off-line analysis, data were played back on a chart recorder with a frequency response of DC-25 kHz (Astro-Med DASH IV, Warwick, RI).

Drug concentrations and method of application

γ-Aminobutyric acid (GABA) was prepared as a 10 mM stock solution dissolved in the HEPES extracellular solution. Clonazepam first was dissolved in dimethyl sulfoxide (DMSO) at 100 mM and then diluted in HEPES to the final concentration. The maximum concentration of DMSO used in cellular perfusion was <0.001%, with application of DMSO alone (0.1%) not altering GABA responses. The applied drug concentrations were as follows: 1–1,000 μM GABA, 0.1–100 nM clonazepam, and 1–300 μM picrotoxin (all from Sigma, St. Louis, MO). Solution changes were accomplished using a modified 13 barrel “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 pattern. Rapid (50–200 ms) and complete solution changes at a constant flow rate then were effected by moving the tube assembly laterally in relation to the neuron under study with no cross contamination evident between tubes. After breaking the seal and allowing ~2–5 min to pass to establish stable leak currents (0 to ~200 pA), GABA was applied for 5–6 s and washed out with control external solution for 30–40 s, all at a holding potential of ~24 mV. The cell was pretreated with test drugs without GABA solutions for 50–60 s and then test solutions were applied together with GABA. Clonazepam drug effects were expressed as percentage augmentation of control 10 μM GABA-evoked outward currents, recorded at a V_HOLD of ~20 mV. Experiments were performed at room temperature (22–24°C).

Current density

Current density was calculated based on the maximal response of a neuron to GABA 1 mM, which then was divided by the membrane capacitance of the cell, read directly off the capacitance compensation potentiometer on the patch amplifier, which, assuming a capacitance/membrane area relationship of 1 μF/cm^2, should be proportional to membrane area.

Statistics

All data were analyzed by calculating the current amplitude of test solutions relative to currents evoked by GABA application alone, and only reversible effects were analyzed with all data expressed as means ± 1 SE. Both the GABA concentration/response and the clonazepam concentration/GABA augmentation curves were fitted by the Marquardt-Levenberg nonlinear least squares method. The significance of differences in fit parameter values...
between curves was assessed using constrained simultaneous curve fitting testing the equality of parameters, and ALLFIT, as described in De Lean et al. (1987). This method involves testing for equality of parameters by examining the statistical consequences (via an F test) of forcing them to be equal.

RESULTS

Characteristics of the low Mg²⁺ hippocampal culture model of epileptogenesis

Intracellular current-clamp recordings from cultures grown and recorded in media containing normal levels of Mg²⁺ exhibited modest levels of spontaneous activity, with frequent spontaneous excitatory and inhibitory synaptic potentials that sometimes elicited individual action potentials (Fig. 1A). During the 3-h low Mg²⁺ extracellular exposure, neuronal firing behavior changed from occasional individual action potentials to spontaneous, continuous, high-frequency action potential firing (Sombati and DeLorenzo 1995). After the readdition of extracellular Mg²⁺ after 3 h of low Mg²⁺ treatment, the tonic, high-frequency discharge activity ceased. However, a permanent “epileptic” alteration in electrophysiological behavior remained, consisting of spontaneous bursting reminiscent of recurrent paroxysmal depolarizing shifts recorded in epileptic preparations both in vivo and in vitro (e.g., Coulter and Lee 1993; Schwartzkroin and Prince 1978; Traub and Wong 1988), interspersed with prolonged (20-s to 2-min duration) seizure-like depolarizations with overriding multispike epileptic discharges, which ranged in frequency from 3 to 20 Hz during the seizure-like event, as described previously (Sombati and DeLorenzo 1995) (Fig. 1B). The majority of epileptic seizures were 20–40 s in duration, but some were ≈2 min in duration. This epileptic neuronal activity has been shown previously to be a population phenomenon using paired recordings and intracellular calcium fluorescence measurements, which con-

FIG. 1. Induction of epileptiform discharge activity in cultured hippocampal neurons. A: a representative intracellular recording from a control neuron showing spontaneous excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs) and occasional spontaneous action potentials. Expansion trace (right) illustrates a faster speed expansion of activity indicated (•••). Resting potentials of individual neurons ranged from −50 to −65 mV. B: a representative intracellular recording from an “epileptic” neuron obtained after a 3-h Mg²⁺-free treatment, showing 4 electrographic seizure discharges arising and ceasing spontaneously. Seizure is shown at faster sweep speeds to demonstrate progressive depolarization at beginning of each episode, which initiated burst discharges of increasing amplitude and accelerating frequency. Further expansion of seizure discharge illustrates numerous action potentials associated with each depolarization.
firmed simultaneous cyclical elevations of intracellular calcium concentrations in large populations of neurons in a time scale exactly overlapping that is seen in intracellular recordings of seizure-like epileptiform activity (Sombati and DeLorenzo 1995).

Hippocampal neuronal cultures appeared similar in morphology and cellular density when preexposed to the low Mg\(^{2+}\) media or to normal levels of extracellular Mg\(^{2+}\) (Fig. 2). Extensive dendritic arborizations with no noticeable differences in dendritic morphology or length or alterations in gross soma morphology were observed in both control and epileptic cultures. The photomicrographs in Fig. 2 show the same field at different time points in normal Mg\(^{2+}\) media before epileptogenic treatment, 1 day post low Mg\(^{2+}\) treatment, and 3 days posttreatment. There were no noticeable differences in cell density or morphology of individual neurons in this culture accompanying the transition of the culture to a chronic epileptic condition characterized by recurrent spontaneous epileptiform activity. Hippocampal neurons were selected for electrophysiological recording in an unbiased manner in both control and low Mg\(^{2+}\) cultures. Only medium to large pyramidal shaped cultured hippocampal neurons were recorded, using identical criteria in both control and treatment conditions. Bipolar and multipolar neurons or neurons that were not overtly pyramidal in shape were not recorded. There were no grossly noticeable differences

![Photomicrographs of normal and low Mg\(^{2+}\) pretreated hippocampal neurons in same field at different time points.](http://jn.physiology.org/)

**FIG. 2.** Photomicrographs of normal and low Mg\(^{2+}\) pretreated hippocampal neurons in same field at different time points. A: neurons treated with normal Mg\(^{2+}\) media. Note extensive dendritic arborizations present in neurons in culture. B: neurons 1 day after low Mg\(^{2+}\) treatment. C: neurons 3 days after low Mg\(^{2+}\) treatment. Note that there were no noticeable gross anatomic differences between neurons in a normal Mg\(^{2+}\) media and neurons preexposed to a media containing no added Mg\(^{2+}\), despite the fact that this treatment resulted in generation of spontaneous recurrent epileptiform activity, which persisted for life of neurons in culture. Calibration bar represents 100 μm.
or alterations in soma or dendritic morphology or cellular density in control and low Mg\(^{2+}\) treated neurons, and no evidence of cell swelling, even in cells within 10 min of the low Mg\(^{2+}\) treatment, where acute effects of the treatment would be expected to be maximal. Membrane capacitance in control neurons was 35.1 ± 1.1 pF (mean ± SE, n = 7), compared with 30.2 ± 2.0 pF in low Mg\(^{2+}\) exposed neurons 10 min after treatment (n = 5) and 45.6 ± 4.0 pF at 2 h after treatment (n = 6), 48 ± 6 pF 1 day posttreatment (n = 5), 33.1 ± 4.3 pF 2 days posttreatment (n = 5), 45.8 ± 3.6 pF 3 days posttreatment, and 50.1 ± 4.2 pF 8 days posttreatment (n = 5). There was no statistically significant difference in the capacitance of these populations of neurons, even at the 10-min time point, when acute swelling effects of the low Mg\(^{2+}\) treatment would be expected to be maximal if present (P > 0.05; 1-way analysis of variance). In contrast to the low Mg\(^{2+}\) treatment, glutamate exposure-induced excitotoxicity causes acute cell swelling and delayed massive neuronal death (Choi 1987; Rothman et al. 1987). In the present study, to examine the issue of potential excitotoxicity engendered by the experimental treatment, neuronal counts showed that the low Mg\(^{2+}\) treatment induced a 6.8 ± 0.6% (n = 3 cultures) loss 48 h after exposure compared with control cultures. This modest level of low Mg\(^{2+}\)-induced neuronal loss contrasts significantly with glutamate exposure studies where 90–100% loss of neurons is observed within 48–72 h after exposure (Choi 1987).

**Reduced postsynaptic GABA\(_A\) receptor current density associated with epileptogenesis**

Conversion of the culture to the epileptic state by low Mg\(^{2+}\) pretreatment induced a significant decrement in the postsynaptic response to increasing concentrations of exogenously applied GABA (1–1,000 μM) (Figs. 3, 4, and 6). Hippocampal culture neurons were voltage-clamped at a V\(_{HOLD}\) of −24 mV, providing a driving force of −46 mV from the theoretical E\(_{GABA}\) of −70 mV, as calculated by the Goldman-Hodgkin-Katz equation for a chloride conductance (Goldman 1943; Hodgkin and Katz 1949), assuming a phosphate 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 response to exogenous GABA application was entirely due to activation of GABA\(_A\) receptors because the intracellular electrode solution contained no potassium, which would preclude outward movement of potassium ions; the reversal of GABA-activated conductance was identical to that predicted for a chloride conductance using the Goldman-Hodgkin-Katz equation (data not shown) (see Gibbs et al. 1996a; Oh et al. 1995) and the response was blocked completely by picrotoxin at higher concentrations (see below) (Coulter et al. 1990). In hippocampal pyramidal neurons in all cultures, the GABA response always was found to increase as increasing concentrations of GABA were applied to cultured hippocampal neurons. The effect of increasing concentrations of applied GABA was found to peak at 1 mM as has been seen in acutely isolated rat (Gibbs et al. 1996a; Oh et al. 1995) and human cortical neurons (Gibbs et al. 1996b,c). GABA concentration response curves were fitted using a nonlinear least squares method assuming a monophasic sigmoidal GABA concentration/response relationship with the equation

\[ I = I_{max}C^n/(C^n + EC_{50}) \]

where C is the GABA concentration, I is the current elicited by a given GABA concentration normalized to the GABA\(_A\) current elicited by application of GABA 1 mM in the same neuron, I\(_{max}\) is the maximal GABA\(_A\) current expressed as a percentage of the GABA 1 mM response, EC\(_{50}\) is the GABA concentration eliciting half-maximal current, and n is the Hill coefficient. A significant reduction in postsynaptic GABA\(_A\) receptor current density was observed as rapidly as 2 h after low Mg\(^{2+}\) treatment (Fig. 3), but not at a 10-min time point during low Mg\(^{2+}\) treatment (before the exchange to normal medium, Fig. 5). The amplitude of GABA\(_A\) responses to GABA 1 mM were reduced ~50% in low Mg\(^{2+}\) treated neurons compared with control cultures. Similar changes in the efficacy of GABA were measured in epileptic cultures 1–4 and 8 days after low Mg\(^{2+}\) pretreatment, associated with similar (50–65%) decrements in postsynaptic functional GABA\(_A\) receptor current density (Figs. 4 and 5). At each time interval after low Mg\(^{2+}\) pretreatment, GABA\(_A\) receptor current density was decreased significantly in comparison with controls and stayed depressed for the life of the culture (Fig. 5). The current density decreased from 2.15 ± 0.27 pA/μm\(^2\) (n = 8) in normal Mg\(^{2+}\) media to 1.03 ± 0.22 pA/μm\(^2\) (n = 6) 2 h after low Mg\(^{2+}\) treatment, 1.16 ± 0.27 pA/μm\(^2\) (n = 5) 1 day after treatment, and to a final GABA\(_A\) postsynaptic current density of 0.75 ± 0.19 pA/μm\(^2\) (n = 5) 8 days after low Mg\(^{2+}\) treatment. Additionally, current density was determined after only 10 min of low Mg\(^{2+}\) treatment to examine the short-term effects of low-Mg\(^{2+}\) treatment (n = 4). No difference was observed between the 10-min low Mg\(^{2+}\) treatment and the control cultures. This short 10-min low Mg\(^{2+}\) treatment was not associated with the development of spontaneous epileptiform activity.

**Postsynaptic GABA\(_A\) responses**

To assess whether the epileptogenesis-associated reductions in functional GABA\(_A\) receptor current density were accompanied by alterations in the properties of the residual receptors compared with controls, GABA concentration/response curves were plotted for data obtained in response to GABA application in concentrations ranging from 1 to 1,000 μM to cultured hippocampal neurons at various time points after the low Mg\(^{2+}\) treatment. To examine the potency of GABA in activating GABA\(_A\) receptors as it potentially varied across the various treatments, all data were normalized to the GABA response to GABA 1 mM (so as to directly compare potencies in differing efficacy responses). Curves were fitted kinetically using the equation presented above. The potency or EC\(_{50}\) of GABA did not change after low Mg\(^{2+}\) treatment and the induction of epileptogenesis (Fig. 6). Control GABA-evoked responses showed an EC\(_{50}\) of 4.5 ± 0.2 μM (n = 8; mean ± SE), whereas low Mg\(^{2+}\) cultures had EC\(_{50}\)s of 3.5 ± 0.1 (n = 6), 5.2 ± 0.5 (n = 6), 3.7 ± 0.3 (n = 6), and 4.6 ± 0.3 μM (n = 5) for 2 h, 2 days, 3 days, and 8 days after treatment, respec-


**Effects of clonazepam on GABA-induced Cl⁻ currents**

Cultured hippocampal neurons were voltage-clamped at −24 mV, and GABA 10 μM was applied alone and concurrently with varying concentrations of clonazepam (CNZ) to examine CNZ augmentation of GABAᵦᵥ-evoked chloride currents. This concentration of GABA was on the rising phase of the concentration/response curve and produced minimal desensitization (Gibbs et al. 1996a; Oh et al. 1995). Application of CNZ in concentrations from 0.1 to 100 nM resulted in a concentration dependent, sigmoidally increasing potentiation of GABAᵦᵥ current amplitude in control cultures. The equation used to fit the CNZ concentration/GABA augmentation curve was

\[ \% \text{ Augmentation} = \frac{M_1 C^H}{(C^H + EC_{50}^H)} \]

where \( M_1 \) is the maximal CNZ effect, \( C \) is the concentration of CNZ, \( H \) is the Hill coefficient, and \( EC_{50} \) is the CNZ concentration at which half-maximal effect is seen. Clonazepam augmentation of GABA responses virtually was abolished in hippocampal cultures 10 days after low Mg²⁺ pretreatment (Fig. 7). The calculated values of \( M_1 \) for CNZ augmentation of GABA responses varied between normal and low Mg²⁺ treated neurons (Fig. 7). The efficacy (\( M_1 \)) of CNZ decreased from 44.1 ± 5.0% (\( n = 10 \)) in control cultures to 9.3 ± 3.2% (\( n = 6 \)) in hippocampal cultures 10 days after low Mg²⁺ pretreatment.

**Picrotoxin induction of epileptic discharges**

Additional experiments were conducted to determine the potential relationship between the reduction in GABAergic efficacy associated with epileptogenesis in epileptic cultures and the actual mechanisms responsible for generation of
ALTERED GABA RECEPTORS IN EPILEPTIC LIMBIC CULTURES 2145

FIG. 4. GABA responses in control and 1–3 and 8 days after low Mg²⁺ pretreatment hippocampal neurons. A: traces of GABA responses in a control and 1–3 and 8 days after low Mg²⁺ treatment neurons illustrating persistent decrement in efficacy of GABA in these “epileptic” cells relative to controls, when GABA is applied at concentrations of 1–1,000 μM. B: GABA concentration/response curves of control and 1–3 and 8 days after low Mg²⁺ pretreatment neurons. Note that concentration response curve for 1–3 and 8 days after low Mg²⁺ neurons show a 50–65% reduction in efficacy of GABA, with no difference in potency in relation to control neurons.

the epileptic discharges. Induction of the epileptic state was associated with a significant reduction (50–65% at GABA 1 mM) in the amplitude of GABA-evoked responses (Figs. 3–5). Experiments were designed to examine whether a similar or smaller level of reduction in GABAergic efficacy in normal cultures also would be associated with the occurrence of recurrent spontaneous seizurelike events, as might be expected if the GABAergic alterations were wholly or partially responsible for the spontaneous epileptiform activity. To do this, a noncompetitive GABA_A antagonists, picrotoxin, was chosen for study, because the efficacy of this drug is independent of the amount of GABA present in the cultures, and the level of GABA blockade by picrotoxin therefore could be controlled rigorously. A concentration/response relationship for picrotoxin inhibition of GABA responses was first determined in the hippocampal cultures under study (Fig. 8). This is an important study to conduct, because agonist and antagonist effects on the GABA receptor are determined to a great extent by the subunit composition of the GABA receptor (e.g., Lüddens and Korpi 1995; reviewed in Macdonald and Olsen 1994), which can vary dramatically in different brain areas and under different culture conditions. Kinetic fits of the picrotoxin concentration response relationship provided a picrotoxin IC₅₀ of 14 μM (n = 7) in cultured hippocampal neurons under the present experimental conditions. Application of picrotoxin at half this concentration (7 μM) was sufficient to block 30% of the GABA response in hippocampal cultures (Fig. 8). This concentration of picrotoxin was also found to be sufficient to trigger spontaneous recurrent epileptiform activity indis-
the reduction of postsynaptic GABA$_A$ inhibition associated with epileptogenesis in the low Mg$^{2+}$ model could be a decrease in the functional expression of GABA$_A$ receptor subunits. Decreases in the mRNA levels of GABA receptor subunits, in particular the $\alpha_2$ and $\alpha_5$ subunits, previously have been seen acutely with the induction of epileptogenesis in a hippocampal slice model exposed to low Mg$^{2+}$ (Vick et al. 1996), chronically in a pilocarpine model of chronic

tinguishable from that seen in low Mg$^{2+}$ pretreated epileptic cultures (Fig. 9). Therefore, the 50–65% reduction of GABAergic efficacy measured in these cultures was sufficient to explain the occurrence of spontaneous seizure-like activity, although other processes certainly could contribute to the generation of these discharges.

**DISCUSSION**

In this study, whole cell voltage-clamp techniques were employed to analyze the potential role of alterations in postsynaptic GABA$_A$ currents in epileptogenesis in cultured hippocampal neurons transformed to a chronic epileptic state by pretreatment with low Mg$^{2+}$ extracellular media. Cultured hippocampal neurons exposed to normal and low Mg$^{2+}$ extracellular levels appeared similar in morphology and cellular density and showed identical sensitivities and EC$_{50}$S to exogenous GABA application. However, neurons recorded from epileptic cultures showed a marked reduction in the current density of functional GABA$_A$ receptors and a diminished modulation of GABA responses by clonazepam. The reduction in postsynaptic functional GABA$_A$ receptor current density and altered GABAergic pharmacology induced by an epileptogenic treatment could have resulted from several possible mechanisms, including alterations in GABA$_A$ subunit expression, GABA receptor downregulation, or post-translational modification of the GABA$_A$ receptor complex, with none of these possibilities being mutually exclusive.

One important possible mechanism that could account for

![Figure 5](image)

**FIG. 5.** Time series of GABA current density illustrating decrease induced by 3-h pretreatment with a low Mg$^{2+}$ media. Note that there was no effect after application of low Mg$^{2+}$ for 10 min ($\rightarrow$). Three-hour low Mg$^{2+}$ pretreatment induced an ~50–65% decrease in GABA current density as soon as 2 h after treatment (*$P < 0.001$). Reduced GABA current density was sustained for life of culture (measured out to 8 days after treatment).

![Figure 6](image)

**FIG. 6.** GABA concentration response curves of control and low Mg$^{2+}$ pretreated neurons. A: GABA concentration response curves in control and 2 h after low Mg$^{2+}$ treated neurons, normalized to GABA response evoked by 1 mM GABA (maximal response). Note that there was no difference in EC$_{50}$ between 2 groups of neurons. B: GABA concentration response curves in control, 2-, 3-, and 8-day after low Mg$^{2+}$ treated neurons, normalized as in A. Note there was no difference in EC$_{50}$ between controls and treatment groups of neurons ($P > 0.05$; ALLFIT).
temporal lobe epilepsy (Houser et al. 1995; Rice et al. 1996), and chronically in cultured hippocampal neurons pre-exposed to low Mg\(^{2+}\) media (Blair et al. 1996). In the pilocarpine model, \(\alpha_2\) and \(\alpha_3\) subunit mRNA levels remained permanently downregulated in the CA1 region of the hippocampus for as long as the rats manifested recurrent seizure discharge activity, but no changes were seen in \(\alpha_1\), \(\beta_2\), and \(\gamma_2\) subunit expression (Rice et al. 1996). Importantly, the selective decreases in mRNA expression did not correlate with neuronal cellular loss in the pilocarpine model (Houser et al. 1995; Rice et al. 1996). These findings suggest that epileptogenesis may cause a selective decrease in the genetic expression of specific GABA\(_A\) receptor subunits. Additional data suggest that this GABA downregulation may be an important mechanism responsible at least in part for triggering the epileptic activity evident in these cultures. In the hippocampal culture model, a selective knockdown of the \(\alpha_2\) subunit with antisense oligonucleotides induced recurrent epileptic seizure discharge activity, whereas removal of the antisense \(\alpha_2\) oligonucleotide resulted in a gradual decline of the seizure discharge activity (Jakoi et al. 1996). A similar induction of recurrent epileptic seizure discharge activity has been obtained with \(\alpha_3\) antisense oligonucleotide knockdown (E. R. Jakoi, S. Sombati, and R. J. DeLorenzo, unpublished data). The association of the decrease of GABA\(_A\) receptor subunit mRNA and the subsequent loss of the inhibition in

![Figure 7](image-url)
the low Mg²⁺ model implies that decreased receptor availability may contribute to the loss of inhibition, which could in turn contribute to increased excitability, triggering the epileptiform discharges as observed in the this model of epilepsy.

Other possible mechanisms could be hypothesized potentially underlying the reduced GABA current density and altered pharmacology evident in neurons recorded after low Mg²⁺ treatment. If this treatment induces either prolonged cell swelling or significant neurotoxicity, then the potential exists that the composition of the medium to large pyramidal cell populations recorded before and after treatment were fundamentally different. The pyramidal cell population that was recorded before treatment could have been depleted significantly due to cell death, and a different population of cells sampled posttreatment, with this latter population having different GABA receptors. A second possibility is that prolonged cell swelling may have occurred after low Mg²⁺ treatment, which would reduce artificially the recorded GABA current density by increasing cell capacitance in the presence of unchanging numbers of GABA receptors. One would expect that if either of these mechanisms were occurring, significant cell death or capacitance changes should be evident in the treatment cultures compared with controls. Only modest levels of cell death were evident in treated cultures (6.8% on average), and no significant differences in cell capacitance were recorded at any time point after low Mg²⁺ treatment, even at the 10-min time point, where these cell swelling effects would be expected to be maximal. So, although the potential for these kinds of “anatomic” shifts in the recorded populations still must be considered, the probability that these types of effects account fully for the large amplitude shifts in both GABA current density and benzodiazepine pharmacology alterations seem unlikely.

The EC₅₀s for GABA activation of GABAₐ currents in cultures that were exposed to normal or low Mg²⁺ media showed no statistically significant difference. This differs from previous findings in other studies of altered GABA responses associated with seizures, where decreases in both GABAergic potency and efficacy were seen in acutely isolated CA1 neurons as an acute consequence of pilocarpine-induced status epilepticus compared with control rats (Kapur and Coulter 1996) and as a consequence of prolonged epilepsy partialis continua in human cortical neurons isolated
from a patient with Rasmussen's Encephalitis (Gibbs et al. 1996b) compared with control GABA responses from non-epileptic (i.e., nonfocal) human cortical neurons (Gibbs et al. 1996c). In contrast to the lack of effect of low Mg\(^{2+}\) pretreatment on the potency of GABA, reductions of functional GABA\(_A\) receptor current density were evident as soon as 2 h after low Mg\(^{2+}\) pretreatment (Figs. 3–5). This low Mg\(^{2+}\) pretreatment induces continuous epileptiform high-frequency discharges, which could be equated with status epilepticus (Sombati and DeLorenzo 1995). GABA levels in the in vivo pilocarpine model have been shown to rise early and to remain elevated in the late stages of pilocarpine-induced status epilepticus (Walton et al. 1990). If a similar increase in GABA levels occurs in the cultures as a consequence of low-Mg\(^{2+}\)-induced status epilepticus, one possibility is that increased levels of endogenous extracellular GABA in the hippocampal cultures resulting from the low Mg\(^{2+}\) status epilepticus discharges could have induced a downregulation in GABA\(_A\) receptors or subunits, which in turn could result in a functional reduction of the postsynaptic current density of GABA\(_A\) receptors in the low Mg\(^{2+}\) treated cultures. Alternatively, acute posttranslational modulation of the GABA\(_A\) receptor may occur during prolonged epileptic events to reduce the number of available GABA\(_A\) receptors. Elevated intracellular calcium concentrations induced by the prolonged seizure discharges occurring as a result of low Mg\(^{2+}\) treatment could activate phosphatases or inhibit selected protein kinases and alter the phosphorylation state of GABA receptors in the hippocampal cultures. Decreased phosphorylation of GABA\(_A\) receptors has been shown to lead to a reduction in GABAergic function (Stelzer et al. 1988). This alteration in phosphorylation state could under-

**FIG. 9.** Current clamp recordings in control and picrotoxin-treated neurons. A: an intracellular recording from a control neuron showing EPSPs and IPSPs and occasional spontaneous action potentials. An expansion at a faster sweep speed illustrates spontaneous synaptic activity associated with control cultures. B: a recording from a neuron treated with bath applied picrotoxin 7 \(\mu M\) illustrating induction of spontaneous epileptiform bursting activity. Note that this concentration of picrotoxin (7 \(\mu M\)) was sufficient to reduced GABA-evoked currents by 30% (see Fig. 8), which was less than GABA reductions induced by the low Mg\(^{2+}\) treatment (>50%, Figs. 3–5). Thirty percent reduction in GABA current was sufficient to induce spontaneous epileptiform activity indistinguishable from that seen in low-Mg\(^{2+}\) pretreated "epileptic" cultures. Individual complex burst firing events are shown at a faster sweep speed to further illustrate morphology associated with epileptiform activity induced by picrotoxin medium.
lie some of the acute effects of low Mg\(^{2+}\) treatment on functional GABA receptor levels (e.g., the reduction seen 2 h after treatment, Figs. 3 and 5). Whether this could account for long-term changes (>1 wk) in GABA receptor levels remains to be determined.

The concept of an epilepsy-associated decrease in inhibition forms the basis of the “GABA hypothesis of epilepsy,” which posits that decreases in the strength of GABA-mediated inhibitory neurotransmission associated with epilepsy leads to an imbalance favoring excitatory transmission and results in the occurrence of epileptic seizure discharge activity in the brain (reviewed in De Deyn et al. 1990). Although this hypothesis is supported by the fact that blockade of GABA transmission by antagonists such as bicuculline and picrotoxin can induce acute pathological burst firing behavior and epileptic discharges, that GABA agonists or GABA metabolism inhibitors are clinically effective anticonvulsants, and that some indicators of levels of GABA function indicate potential impairments in patients with temporal lobe epilepsy (De Deyn et al. 1990; During et al. 1995; Johnson et al. 1992; Savic et al. 1988). There has been a paucity of data amassed to date in chronic epilepsy models (i.e., models in which spontaneous recurrent seizure discharges are generated in normal medium), which demonstrate a sustained attenuation of inhibitory transmission underlying and generating epileptogenesis. In addition, models exist that do not manipulate the GABAergic system (e.g., low Mg\(^{2+}\) perfusion, high K\(^{+}\) perfusion) but still result in pathological epileptiform activity (e.g., Coulter and Lee 1993; Traynelis and Dingledine 1988; Walther et al. 1986; Wilson et al. 1990; Zhang and Coulter 1996; Zhang et al. 1996a,b).

Few models of epilepsy to date have unequivocally and convincingly demonstrated both acute and long-term decreases in GABAergic inhibition that could underlie development of a permanent epileptic state. In the kindling model, mRNA levels of several GABA receptor subunits vary acutely during the kindling process in the dentate gyrus, but return to control levels within a few days, suggesting that reductions in the genetic expression of GABA receptors are not involved in maintenance of the epileptic condition (Kamphius et al. 1995; Kokia et al. 1994). In fact, long-term elevated levels of expression of both α\(_2\) and γ\(_2\) GABA subunit mRNA were evident in the dentate gyrus of kindled rats, suggesting, if altered at all, GABAergic inhibition actually might be enhanced in the dentate gyrus by kindling (Kamphius et al. 1995). Recordings of spontaneous inhibitory postsynaptic currents in dentate gyrus of kindled rats have shown these events to be larger in epileptic animals than in controls, and this amplitude increase was attributed to increased numbers of postsynaptic GABA receptors present in granule cells of epileptic animals (Otis et al. 1994). Further electrophysiological evidence for alterations in the functional composition of dentate gyrus GABA receptors has suggested that this kindling-associated elevation in inhibition may collapse due to enhanced zinc sensitivity of GABA responses in kindled animals. It is hypothesized that zinc release during sustained synaptic activation will act dynamically to block inhibition during pathological excitation in the dentate gyrus of kindled animals (Buhl et al. 1996) and recently in the pilocarpine model of epilepsy (Gibbs et al. 1997). Evidence also has been presented suggesting that functional deafferentation of interneurons in CA1 of hippocampus may occur as a long-term consequence of kindling, resulting in decreases in feedforward and feedback inhibition in this hippocampal subfield independent of alterations in postsynaptic GABA receptors (Bekenstein and Lothman 1993). The low Mg\(^{2+}\) culture model demonstrates not only an acute attenuation but also a chronic, prolonged decrease in functional GABAergic inhibition that has not been reported previously in a model of epilepsy. The noncompetitive GA\(_B\)_A receptor blocker, picrotoxin, was used to mimic the effects observed after treatment with a low Mg\(^{2+}\) media to examine the potential role of epileptogenesis associated reductions in GABA-mediated transmission in generation of the electrophysiological epileptic behavior of the hippocampal culture model. Bath application of picrotoxin 7 μM was sufficient to induce spontaneous epileptiform activity indistinguishable from that seen in the epileptic cultures (Fig. 9). This concentration of picrotoxin blocked only 30% of the GABA current (Fig. 8), whereas there was a 50–65% reduction of the GABA-evoked responses in the epileptic cultures (Figs. 3–5). This suggests that the level of reduction of GABA responses evident in the epileptic cultures was more than sufficient to underlie the generation of spontaneous epileptiform activity.

Cultured hippocampal neurons exposed to a low Mg\(^{2+}\) pretreatment also exhibited diminished GABAergic modulation by clonazepam. Reductions in GABA\(_A\) modulation by benzodiazepines as a result of the induction of spontaneous, epileptiform discharge activity has important clinical implications, because loss of GABAergic modulation by anticonvulsants such as clonazepam could change clinical treatment strategies. A decrease in the functional expression of the γ subunit, which confers benzodiazepine sensitivity to the GABA\(_A\)_A receptor complex (reviewed in Macdonald and Olsen 1994), or the α subunit, to which the benzodiazepine binds and the nature of which determines the pharmacology of the benzodiazepine response, could be downregulated, resulting in a decreased pharmacological efficacy to benzodiazepines (Prichett et al. 1989). Decreased efficacy of benzodiazepines has been reported as soon as 35 min after onset of seizures in status epilepticus (Walton and Treiman 1988).

In another epilepsy model, decreased benzodiazepine binding has been demonstrated 1 mo after initial kindling in the CA1 and CA2 regions of hippocampus (Shin et al. 1985; Titulaer et al. 1995a,b). In the pilocarpine model of epilepsy, a decrease in the efficacy of benzodiazepines was observed in CA1 neurons (Gibbs et al. 1997). In the low Mg\(^{2+}\) culture model, decreased expression of the α\(_2\) and α\(_5\) subunits were observed (Blair et al. 1995). The presence of these subunits within a GABA receptor pharmacologically confer type II benzodiazepine characteristics (reviewed in Macdonald and Olsen 1994). A decrease in the overall number of GABA\(_A\) receptors or function brought about by subunit downregulation after periods of intense and prolonged epileptiform activity, such as status epilepticus, would render benzodiazepines less efficacious. This could help explain the increased difficulty seen in the treatment of prolonged human status epilepticus, where insensitivity to benzodiazepines is often seen (Treiman et al. 1990). In human neurons acutely isolated from a patient with Rasmussen’s Encephalitis, efficacy of clonazepam (100 nM) in modulating GABA responses
also was reduced significantly (Gibbs et al. 1996b) relative to nonepileptic control cells (Gibbs et al. 1996c).

The presently described hippocampal culture model of epilepsy provides a potentially powerful and useful tool to study mechanisms associated with the development of chronic recurrent spontaneous seizure discharge activity characteristic of the epileptic condition. Currently, few in vitro models exist that offer equivalent superior access and long-term viability necessary to study the biophysical, biochemical, and molecular genetic mechanisms associated with and underlying the generation of spontaneous recurrent seizure discharge activity such as that evident in this model. There are also several distinct disadvantages to this culture model. One disadvantage that immediately comes to mind is that there are no characteristic anatomic pathways present in dispersed cultures, making stimulation of pathways and recognition of cell types impossible, beyond limiting attention to medium to large pyramidal neurons. A second potential disadvantage is that it is unknown whether normal development of hippocampal neurons is recapitulated fully in vitro. Important factors critical in epileptogenesis in vivo may not develop in vitro or might overdevelop. These disadvantages can be accounted for experimentally by constantly cross-fertilizing and comparing results from this culture model with results from in vivo models such as kindling and chemovoncussant-induced epileptic conditions, as well as in vitro models.

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