Burst Generation in Neocortical Neurons
After GABA Withdrawal in the Rat

C. SILVA-BARRAT, S. ARANEDA, CH. MENINI, J. CHAMPAGNAT, AND R. NAQUET
Laboratoire de Physiologie Nerveuse, C.N.R.S., 91198 Gif-sur-Yvette, France

SUMMARY AND CONCLUSIONS

1.  g-Aminobutyric acid (GABA) withdrawal syndrome (GWS) represents a particular model of focal epilepsy consecutive to the interruption of a chronic intracortical GABA infusion and is characterized by the appearance of focal epileptic electroencephalographic (EEG) discharges and localized clinical signs on withdrawal of GABA. Effects of Ca2+ channel blockers and N-methyl-D-aspartate (NMDA) antagonists were evaluated in living rats presenting a GWS after interruption of a 5-day GABA infusion into the somatomotor cortex and in neocortical slices obtained from such rats. Bursting properties and morphology of neurons were also analyzed in slices.

2.  In living rats, the noncompetitive NMDA antagonist phenytoyclicline [1-((1-phenylcyclohexyl)piperidine] and the Ca2+ antagonist flunarizine [E-1(bis(4fluorophenyl)methyl)-4(3phenyl-2-propenyl)-piperazine] were administered systematically to two groups of rats. Rats in the first group (n = 12) were injected with the drug 30-60 min before discontinuation of the GABA infusion. In this case, phenytoyclicline (10 mg/kg ip) prevented the development of GWS (n = 5), whereas flunarizine (40 mg/kg ip) had no consistent effect on the GWS appearance and characteristics (n = 7). Rats in the second group (n = 12) were injected 60-90 min after GABA discontinuation, i.e., during a fully developed GWS. In that case, neither drug suppressed GWS.

3.  Neuronal activities in the epileptic focus were studied in slices with conventional intracellular recording and stimulation techniques. From the 65 neurons recorded, 29 responded with EPSPs and paroxysmal depolarization shifts (PDSs) to white matter stimulation (synaptic bursting or SB cells). Nineteen other neurons presented, in addition to synaptically induced PDSs, bursts of action potentials (APs) induced by intracellular depolarizing current injection (intrinsic bursting or IB cells). The remaining 17 neurons presented no bursting properties to either synaptic stimulation or depolarizing current injection (nonbursting or NB cells).

4.  The recorded neurons were located 0.7-1.2 mm distant from the lesion because of the penetration of the GABA infusion cannula. Intracellular injection of neurons (n = 4) with biocytin or Lucifer yellow revealed that both SB and IB neurons were large, spiny pyramidal neurons localized in layer V of the sensorimotor cortex.

5.  Bath application of the selective antagonist of NMDA receptors DL-2amino-5phosphonovalerate or DL-2amino-7phosphonoheptanate (10-30 μM) reversibly reduced the amplitude (by 25-50%) and the duration (by 20-25%) of PDSs in all cases (n = 17). At the same doses, NMDA antagonists did not affect the EPSPs induced by a stimulation at low intensity in SB (n = 13), IB (n = 4), or NB (n = 6) neurons or 2) the firing patterns induced by intracellular depolarizing current injection in SB, IB and NB cells.

6.  Intrinsic bursts induced in IB neurons were abolished by organic (n = 4) and inorganic (n = 8) Ca2+ antagonists, unmasking a slow depolarization (3-6 mV in amplitude and 60-100 ms in duration) that underlies these bursts. Both the Ca2+-sensitive and the slow Ca2+-independent depolarization were resistant to tetrodotoxin (TTX) application (0.5 μM). The organic Ca2+ antagonist verapamil (20-30 μM) reduced the amplitude of PDSs by 40-70% and suppressed the bursts of APs riding on the depolarizing wave in SB (n = 5) and IB cells (n = 4). The inorganic Ca2+ antagonists Co2+ (2 mM), Mg2+ (2 mM), and Cd2+ (0.5 mM) also reduced the amplitude of PDSs by >50% and blocked the associated bursts in SB (n = 7) and IB cells (n = 8). None of the Ca2+ antagonists affected EPSPs induced in SB, IB, or NB cells by stimulation at low intensity or the EPSP-single AP sequences evoked in NB cells (n = 5) by stronger stimuli.

7.  The effects of Ca2+ antagonists on IB and SB cells were followed by a transitory enhancement of bursting activity as a rebound of excitability at the beginning of the washout of the Ca2+ antagonists (n = 10). Intracellular application of ethylene glycolbis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), a Ca2+ chelator, increased the amplitude and duration of PDSs and favored the appearance of additional APs in both SB (n = 2) and IB neurons (n = 1).

8.  In conclusion, voltage-dependent Ca2+ currents are responsible for bursting of IB pyramidal neurons but not for epileptogenesis. Moreover, NMDA-associated cationic channels are involved in epileptogenesis and at least partly in the induction of PDSs observed during GWS. Simultaneous involvement of several factors in the genesis of bursts explains the refractoriness of GWS to administration of Ca2+ blockers, NMDA antagonists, or GABA agonists in living rats. The results also emphasize the important role of network interconnections between GABA-treated neurons and neurons located outside the infusion site.

INTRODUCTION

A chronic intracortical infusion of γ-aminobutyric acid (GABA) into the motor cortex gives rise, on cessation, to an epileptogenic focus localized in the infusion area. This focus is characterized by continuous electroencephalographic (EEG) discharges at high frequency (1-2 Hz) associated with myoclonic twitches of the contralateral corresponding body territory. These effects do not depend on a previous predisposition to epilepsy: they were observed in photosensitive and photosensitive baboons (Brai1owsky et al. 1987, 1989; Silva-Barrat et al. 1988) and in amygdala-kindled as well as in normal rats (Brai1owsky et al. 1988, 1990; Fukuda et al. 1987). The observed epileptic focus represents a particular model of drug withdrawal with local epileptic manifestations that has been named "GABA withdrawal syndrome" or GWS (Brai1owsky et al. 1988). The clinical correlates of this GWS resemble the epilepsy partialis continua described in human patients (Bancaud et al. 1982; Kojewnikow 1895).

In neocortical slices obtained from rats presenting GWS, numerous burst-generating neurons were recorded in the vicinity of the GABA-infused site (Champagnat et al. 1990;
Silva-Barrat et al. 1989). Neurons were classified according to their response to white matter (WM) stimulation and intracellular current injection:

1) Intrinsic bursting (IB) cells exhibit bursts of action potentials (APs) when intracellularly injected with a depolarizing current. IB cells also present paroxysmal depolarization shifts (PDSs) when synaptically stimulated.

2) Synaptic bursting (SB) cells present PDSs and bursts of APs only when synaptically induced by WM stimulation.

3) Nonbursting (NB) cells present neither synaptic nor IB properties.

Bursting properties of IB cells have been correlated with a decrease of cellular sensitivity to GABA and to a GABA<sub>A</sub> agonist (isoguvacine), caused by the prolonged infusion of GABA. This effect could be related to a calcium influx that may reduce the GABA<sub>A</sub> receptor-mediated inward current and that seems responsible for the bursting properties (Silva-Barrat et al. 1989). However, the roles of Ca<sup>2+</sup> currents and of Ca<sup>2+</sup>-dependent processes in determining bursting patterns have not yet been clarified.

The entry of Ca<sup>2+</sup> ions appears common to all burst generating cells (Pumain et al. 1983; Schwartzkroin and Wyler 1980). In normal or epileptic rats, burst generation is abolished after application of inorganic or organic Ca<sup>2+</sup> antagonists in the hippocampus (Bingmann and Speckmann 1989; Johnston et al. 1980; Wong and Prince 1978, 1981), neocortex (Pockberger et al. 1986; Walden et al. 1986; Witte et al. 1987), and thalamus (Jahnsen and Llinas 1984). Moreover, flunarizine (E-1-[bis(4-fluorophenyl)-methyl]4-(3phenyl2propenyl)-piperazine), a Ca<sup>2+</sup> blocker, possesses anticonvulsant action in living animal models of epilepsy, such as reflex epilepsies including sound-induced seizures in mice or photically induced myoclonic responses in the photosensitive baboon, and generalized epilepsies induced by convulsants (De Sarro et al. 1986) and kindling in rats (Ashton and Wauquier 1979). In humans, flunarizine reduces the incidence of seizures in patients with partial complex epilepsy (Binnie 1989; Binnie et al. 1985; Overweg et al. 1984).

The entry of Ca<sup>2+</sup> depends not only on voltage-dependent Ca<sup>2+</sup> channels but also on receptor-operated Ca<sup>2+</sup> channels. Thus channels activated by N-methyl-D-aspartate (NMDA) receptors cause a large, inward cationic current, including Ca<sup>2+</sup>, as demonstrated in voltage-clamped neurons (Ascher and Nowak 1988; MacDermott et al. 1986; Mody et al. 1988). Furthermore, NMDA antagonists have significant anticonvulsant effects. In vivo, they reduce or suppress audiogenic seizures (Jones et al. 1984), photogenic seizures (Meldrum et al. 1983), drug-induced seizures (Croucher et al. 1982; Czuczwar and Meldrum 1982), and the kindling phenouenon (Peterson et al. 1983), as well as bursts induced by convulsants or electrical stimulation in slices of rat hippocampus (Dingledine et al. 1986; Neuman et al. 1988; Stasheff et al. 1989) and human neocortex (Avoli and Olivier 1987). The noncompetitive NMDA antagonists [phencyclidine (1-[(1-phenylcyclohexyl)piperidine]-like drugs) have antiepileptic properties against electroshock and kindled seizures in rats (McNamara et al. 1988; Sato et al. 1988). They also have therapeutic applications in some human neurological disorders (see Lodge et al. 1989).

The aim of the present study is to analyze the effects of drugs antagonizing the calcium- or NMDA-induced currents on the GWSs in vivo and on electrophysiologically and morphologically identified bursting neurons in neocortical slices obtained from rats presenting the GWS. Evidence has been obtained in favor of the role played by Ca<sup>2+</sup> and NMDA-induced currents in the expression of seizures that characterize GWSs in living rats. It appears that both intrinsic bursts and PDSs are developed by pyramidal neurons localized in layer V of the sensorimotor cortex and that Ca<sup>2+</sup> inward voltage-dependent currents play a primary role in the genesis of bursts. In addition, cationic influx depending on NMDA receptor activation is involved in PDS generation.

**Materials and Methods**

**Animal preparation**

Male Wistar rats weighing 120–150 g were prepared as already described (Brailowsky et al. 1988). Under general anesthesia (ketamine and xylazine mixture), rats were implanted with epidural screws for EEG recordings. They were also implanted with a cannula (0.4 mm diam) in the left somatomotor cortex (2 mm posterior to bregma, 2 mm lateral, and 1 mm depth), allowing the longterm infusion of GABA for 3 days by the use of Alzet osmotic minipumps. A minipump filled with GABA (100 μg/μl, delivery rate 1 μl/h) was placed under the skin of the animal’s back and connected with a catheter to the intracortical cannula. Five days later, GABA infusion was stopped without anesthesia of animals by simply disconnecting the catheter from the cannula. GWS appeared after a mean delay of 20 min (Brailowsky et al. 1988; 1990).

**Pharmacological tests in living rats**

NMDA and Ca<sup>2+</sup> antagonists were administered systemically according to two protocols under continuous EEG control. In a first group of animals, the drug administration preceded the interruption of GABA infusion by 30–90 min; then, the GABA infusion was stopped and the eventual antiepileptogenic effects were observed. In the second group of animals, the drug administration followed the beginning of GWS by 60–90 min, and the eventual anticonvulsant effects were noted. The exact timing of drug administration was chosen according to the delay for reaching peak plasma level. In all cases, EEG and behavioral controls were pursued for ≤8 hours. The effects of drugs on epileptic charge duration, amplitude, and frequency are expressed as a percentage in comparison with data obtained in a group of non-treated rats presenting GWS (Brailowsky et al. 1990).

The Ca<sup>2+</sup> channel blocker flunarizine and the noncompetitive NMDA antagonist phencyclidine were selected because they cross the blood brain barrier more easily than verapamil and competitive NMDA antagonists. Flunarizine was dissolved in 2% hydroxypropyl-β-cyclodextrin and injected at a dose of 40 mg/kg ip, i.e., the highest dose used by De Sarro et al. (1986) in rats for blocking sound-induced seizures. Phencyclidine was dissolved in saline and injected at a dose of 10 mg/kg ip, which is twice the dose used for testing anticonvulsant effects in kindled rats (McNamara et al. 1988).

**Slice preparation**

The in vitro study involved, successively, the chronic intracortical GABA infusion and the development of GWS in vivo according to the method described above, and then the slice preparation, the intracellular recordings, and drug applications. The methods for this in vitro study have already been described (Silva-Barrat et al. 1989), with exceptions that will be noted.
Rats presenting GWS were decapitated without anesthesia for slice preparation. After removal of the cortex under continuous rinsing with cold Krebs-Ringer solution, transverse slices (450 μm) were cut with a McIlwain chopper at the level of the infusion site. After an initial incubation period of ~1 h in a holding chamber, the slices were transferred to the recording chamber and superfused with warm (32 ± 0 °C, mean ± SE) Krebs-Ringer solution composed of (in mM): 124 NaCl, 5 KCl, 1.25 KH2PO4, 1.25 MgSO4, 7 H2O, 2 CaCl2, 26 NaHCO3, and 10 glucose, pH 7.4. The perfusion system allowed an incoming flow of 2–3 ml/min.

**Drug administration**

The organic calcium antagonist verapamil (verapamil hydrochloride, Knoll) and NMDA antagonists APV (DL-2-amino-5-phosphonovalerate, Sigma) and APH (di-2 amino-7-phosphonohaptoanote, Sigma) were dissolved in the Krebs-Ringer solution and added to the perfusion medium through a valve-manifold system without alteration of the perfusion rate. When CoCl2, and added to the perfusion medium through a valve-manifold system without alteration of the perfusion rate. When CoCl2, MgCl2, or CaCl2 replaced CaCl2, NaH2PO4 was omitted and MgSO4 was replaced with MgCl2 to prevent divalent cation precipitation.

**In vitro neuronal recordings**

Intracellular recordings were made from micropipettes filled with 3 M KCl (50–80 MΩ), 4 M potassium acetate, or 2 M potassium methylsulphate (60–120 MΩ). In some experiments, the filling solution contained 250–500 mM ethylene glycol-bis-(β-aminoethanol ether)-N,N′,N′,N′-tetraacetic acid (EGTA), a Ca2+ chelator (Sigma), and 0.5 M potassium acetate (pH 7). Intracellular application of EGTA was performed through the recording electrode either by passive diffusion or by applying depolarizing pulses every 5 s (200 ms, 0.1–1 nA). Membrane potential recordings and intracellular current injections were performed through the Wheatstone bridge of a DC preamplifier allowing capacity compensation. Single stimuli 0.05 ms in duration and 0.5–1.0 mA in intensity were applied to WM with the use of a tungsten concentric bipolar electrode. Care was taken to position the stimulating electrode within the same cortical column as the recorded cell. The stimuli were delivered by a programmable stimulator every 5 s. Voltage transients and injected currents were displayed on an oscilloscope and a thermal arraycorder (Ankersmit) and stored on a digital tape (Biologic) for off-line analysis. The analysis consisted of an analog-to-digital conversion through a Cambridge Electronic Device (CED 1401) and the treatment of digital data by an averaging program (SIGAVR) on a Hewlett Packard computer.

**Morphological identification of neurons**

The micropipettes used for the combined anatomic and electrophysiological study were filled with 4% biocytin (Sigma) dissolved in 50 μl of tris (hydroxymethyl)aminomethane (Tris) buffer (0.05 M) and KCl (0.5 M, pH 7–7.6). Biocytin solution was injected by means of a continuous hyperpolarizing current of 0.5–1 nA and an additional hyperpolarizing current pulse (200–300 ms duration, 1 Hz frequency, and 0.5 nA of intensity), as reported by Horikawa and Armstrong (1988). After biocytin injection, intracellular responses were checked to verify that the micropipette tip was still within the injected cell.

Slices were fixed overnight in 4% paraformaldehyde in phosphate buffer (0.1 M) containing 0.1% picric acid (pH 7.4) at 4°C. Fixed slices rinsed in phosphate buffer were cut in 40-μm sections with a vibratome. Sections were treated with 0.3% H2O2 (20 min at room temperature) and rinsed for 3–10 min. To reveal biocytin-injected cells, sections were incubated during 3–4 h with streptavidin-biotinylated horseradish peroxidase (HRP) complex (Amersham) diluted (1:200) in 0.1% triton x100. Then the peroxidase reaction was developed for 10–20 min with diaminobenzidine (0.05%) and H2O2 (0.005%) in TRIS buffer. Sections were then mounted on slides, counterstained with cresyl violet, and observed on an optic microscope. In some experiments, microelectrodes were filled with the fluorescent dye Lucifer yellow (LiCl 0.1%), and the neuron recorded intracellularly was stained by passing a hyperpolarizing intracellular current of 1–2 nA for 5–10 min. Slices were then transferred to phosphate-buffered paraformaldehyde for 24 h. Labeled cells were examined and photographed with the use of fluorescent microscopy (Leitz Diaplan).

**RESULTS**

**In vivo observations**

Phencyclidine was administered to rats (n = 5, 10 mg/kg ip) 30 min after the appearance of GWS (Fig. 1). One to two hours later, the epileptic discharges were decreased in amplitude by 30–60% and increased in frequency by 50–100%, but they were not blocked for ≥8 h. During this period, animals exhibited ataxia and stereotyped movements, rendering myoclonic twitches unobservable. Phencyclidine was administered to another group of rats (n = 5, 10 mg/kg ip) 30 min before the interruption of GABA infusion, and the EEG recording was then monitored for ≤10 h (Fig. 1). In this case, phencyclidine prevented the appearance of EEG and clinical epileptic signs.

Flunarizine was administered to rats (n = 7, 40 mg/kg ip) 90 min after the appearance of GWS. Two hours later, epileptic discharges showed a decrease in amplitude by 30% and in frequency of 20%, but, as for myoclonic twitches, they were not blocked. No noticeable behavioral changes were observed. Flunarizine was administered to another group of rats (n = 7, 40 mg/kg ip) 90 min before the interruption of GABA infusion, and the EEG recording was monitored for ≤10 h afterwards. In this case, flunarizine did not prevent the appearance of EEG or clinical epileptic signs, although it provoked a 36% decrease in amplitude and a 16% increase in frequency of epileptic discharges.

**Electrophysiological identification of neurons**

Cortical slices were obtained from rats presenting GWS. Because no proper recording could be made close to the site of infusion cannula penetration, cells were recorded at the periphery of this site. This site corresponds to a zone of increased metabolism, as recently observed in the study of local cerebral glucose utilization (Menini et al. 1991). The recorded cells were selected on the basis of stable membrane and APs over 3- to 7-h periods. Cells were identified depending on their response to WM stimulation and to interruption of GABA infusion. Out of 65 selected cells, 29 were SB neurons showing PDSs, 17 were IB neurons showing PDSs and intrinsic bursts, and the remaining 17 were NB cells showing no bursts. The three cell groups were not significantly different (Student's t test) in their resting membrane potential (75 ± 10 mV), input resistance (39 ± 13 MΩ), and AP amplitude (90 ± 10 mV). We confirm here previous observations showing that the number of IB cells is significantly more important in slices obtained from rats presenting GWS (27%) than from control rats infused with saline (7–8%, Silva-Barrat et al. 1989).

PDSs were evoked in SB and IB cells by WM suprathre-
FIG. 1. Effects of PCP in a living rat. EEG recordings before (-60 min) and at different times after (30, 50, and 100 min) interruption of GABA infusion. A: GWS is apparent as EEG epileptic discharges that predominate in infused left somatosensory cortex (L). PCP (10 mg/kg ip) was administered 40 min after GABA interruption and does not block GWS. B: PCP at the same dose was administered 30 min before GABA interruption and prevented the GWS appearance.

FIG. 2. Recordings in a bursting (A) and a nonbursting cell (B) with potassium methylsulfate electrodes in neocortical slices obtained from rats with GWS. A1: PDS triggered in IB cell by threshold WM stimulation, superimposed on an EPSP triggered in the same cell by subthreshold stimulation. A2: spontaneous burst observed in the same cell, resembling triggered PDS. (Em = -70 mV). B1: EPSP triggered in NB cell by subthreshold WM stimulation. B2: single action potential of the same cell, induced by threshold WM stimulation. (Em = -68 mV). Same calibrations in A and B.
shold stimulation with an intensity 1.5-2 times that required for evoking an excitatory postsynaptic potential (EPSP). Synaptic bursts consist of 3-8 APs riding on the large depolarizing wave or PDS (48 ± 19 mV amplitude, 178 ± 62 ms duration, n = 26). EPSPs were obtained by decreasing the stimulus intensity to values subthreshold for AP elicitation (Fig. 2A1). Spontaneous bursts were occasionally (n = 3) observed with a waveform similar to PDSs (Fig. 2, compare A1 and A2). Intrinsic bursts of IB cells induced by depolarizing current injection consisted of 2-4 APs riding on a slow wave (Figs. 4B and 5C). Such bursts were not seen with depolarizing current in SB cells.

In NB cells recorded with microelectrodes filled with either 4 M potassium acetate or 2 M potassium methylsulfate (n = 12), the WM stimulation subthreshold for AP elicitation evoked a synaptic potential that, at the resting membrane potential, was mainly depolarizing (Fig. 2B1). In the same cells, the suprathreshold stimulation evoked a single AP followed by a biphasic event: an initial afterhyperpolarization followed by a depolarizing after-potential and by a slower AHP (Fig. 2B2).

Morphological identification of neurons

Histological observation of slices showed that the recorded cells were impaled at the periphery of the necrotic zone (0.7-1.2 mm) because of cannula penetration and GABA infusion. The necrotic zone extended to layers V and VI and was characterized by numerous Nissl-stained cells and the disappearance of cortical lamination (Figs. 3 and 4). The soma of the biocytin-labeled neurons (n = 2) were localized in layer V (Figs. 3A and 4A). Both cells had a pyramid-shaped soma (12-15 μm) with a basal dendritic arborization, an apical dendrite, and an axon that arose from the basal region of the soma. Both apical and basal dendrites presented numerous spines. Similarly, neurons labeled with Lucifer yellow (n = 2) were pyramid-shaped neurons of layer V.

Even if the labeled neurons showed a similar pyramidal shape, they presented responses characteristic of either SB or IB neurons. Figure 3B presents a neuron electrophysiologically identified as SB, whereas Fig. 4B shows a morphologically similar pyramidal neuron identified as an IB cell. Morphologically identified pyramidal neurons with IB properties have already been described in a normal neocortex (Agmon and Connors 1989; Larkman and Mason 1990, McCormick et al. 1985; Montoro et al. 1988).

Effects of NMDA antagonists

Drugs were added to the perfusion bath after electrophysiological determination of the cellular type (SB, IB, or NB). The effects of calcium or NMDA antagonists described hereafter did not depend on the type of recording electrode used (chloride-free or chloride-filled microelectrodes).

EFFECTS OF NMDA ANTAGONISTS ON BURSTING NEURONS. Competitive NMDA antagonists APV (in 5 SB and 2 IB
neurons) and APH (in 8 SB and 2 IB neurons) were tested at concentrations of 10–50 μM. They decreased the PDS amplitude by 25–50% and the PDS duration by 20–25% (Figs. 5 and 6). After 2–4 min, the number of APs evoked by the stimulus decreased concomitantly with the PDS amplitude. These effects were reversed by washing APV or APH. In the same neurons, the amplitudes (8.3 ± 2.4 mV) and the shapes of EPSPs did not change at a time when the stimulus-induced bursts were reduced or blocked (7.4 ± 2.6, n = 13). Therefore NMDA antagonists, at the doses used, modified bursts but not the synaptic mechanisms underlying the EPSPs evoked by stimulation at a weak strength.

Depolarizing and hyperpolarizing current pulses were used to monitor excitability and input resistance during APV or APH application. No effect was found on the repetitive firing or membrane input resistance in SB neurons (40 ± 9 MΩ before and 38 ± 11 MΩ after the drug application, n = 13). Furthermore, intrinsic properties of IB neurons were not affected by NMDA antagonists (APH, n = 2; APV, n = 2) at doses acting on PDSs (Fig. 5). These results demonstrate that NMDA receptors play a specific role in the induction of PDSs, but not in the classical EPSPs, intrinsic properties, or input resistance of the neuron.

EFFECTS OF NMDA ANTAGONISTS ON NONBURSTING NEURONS. The effects of APV and APH were also analyzed on eight NB cells. Doses of APV or APH ≤100 μM modified neither the EPSPs induced by a subthreshold stimulus nor the EPSP-single-AP sequence induced by a suprathreshold stimulus. The mean amplitude of EPSP measured at resting membrane potential was 8.2 ± 2.6 mV under control conditions and 7.9 ± 2.7 mV after drug application (n = 8), thus indicating that NMDA receptors do not significantly contribute to excitatory synaptic transmission in NB cells, as described in other models (Collingridge et al. 1983; Dingle-dine et al. 1986). When larger doses of APV or APH were used (100–300 μM), EPSP amplitudes were reduced by 30–60% and APs elicited by suprathreshold stimulus were abolished. As for bursting cells, APV and APH did not modify the passive properties of these NB cells.

FIG. 6. Effects of APV on SB neuron. PDS is superimposed on EPSP triggered in the same neuron by a subthreshold WM stimulation (artifact), before (control), during (APV), and after (recovery) APV application (20 μM). $E_m = -68$ mV.
EFFECTS OF APV IN MAGNESIUM-FREE MEDIUM. The effects of APV were also tested in two NB neurons recorded in Mg²⁺-free medium. In this case, MgSO₄ was omitted from the perfusate medium. During the first minutes of perfusion, the synaptic noise increased and spontaneous APs appeared. Five to fifteen minutes after the perfusion onset, the WM suprathreshold stimulation that triggered a single AP during the control period was able to trigger a synaptic burst of 2–3 APs riding on a depolarization wave similar to that observed in SB cells. Spontaneous PDSs similar to those recorded in hippocampal pyramidal cells bathed in Mg²⁺-free medium (Mody et al. 1987) were not observed. PDSs induced in NB neurons in the absence of Mg²⁺ ions were completely blocked after 5–10 min by APV (50 μM) application. During the return to normal Mg²⁺ concentrations, the cells recovered their initial firing pattern. In summary, the effect of APV was much stronger on Mg²⁺-sensitive bursts in NB cells than on PDSs in SB or IB cells. Thus an impairment of the Mg²⁺ ion–NMDA receptor interaction cannot explain PDS generation in bursting neurons.

Effects of calcium antagonists

The effects of organic and inorganic Ca²⁺ channel blockers and of an intracellularly injected Ca²⁺ chelator were evaluated to clarify the influence of Ca²⁺ influx and Ca²⁺ accumulation on the bursting neuronal properties. To demonstrate blockade of intrinsic bursts, we used current intensities 2–5 times higher than in the control.

EFFECTS OF CALCIUM ANTAGONISTS ON BURSTING NEURONS. The effects of verapamil were tested on five SB and four IB neurons. The effects of verapamil were reversible, dose-dependent, and detectable at a dose of 10 μM. At doses of 20–30 μM, verapamil reduced the amplitude of PDSs by 40–70%, and the APs were suppressed after 2–3 min of application (Fig. 7). During verapamil application, the am-

FIG. 7. Effects of verapamil (20 μM) on IB neuron. A: synaptic response triggered by WM suprathreshold stimulation (artifact). B: intrinsic response triggered by an intracellular depolarizing current (0.1 nA, bottom). From top to bottom, responses obtained before (control), 2 min, and 3 min after onset of verapamil application and 15 min after offset (recovery). During verapamil application, intrinsic bursts were not observed with current injection >0.1 nA and <0.5 nA. C: superimposition of synaptic responses, showing successive stages of the blockade by verapamil in the same neuron: before (1), 2 min (2), 3 min (3), and 5 min (4) after the onset of application (Fᵥm = −68 mV).
antagonists suppressed the bursts occurring spontaneously or those induced by depolarizing current injection, unmasking a 3- to 6-mV, 60- to 100-ms depolarizing wave underlying the burst (Fig. 10). When both intrinsic and synaptic bursts were analyzed simultaneously in a given IB neuron, all Ca$^{2+}$ antagonists affected intrinsic bursts before PDS, similar to verapamil.

After offset of verapamil or inorganic Ca$^{2+}$ antagonists, a transient (2-3 min) exaggeration of both synaptic and intrinsic bursting patterns was seen in virtually all neurons. Thus antiepileptic effects were followed, at the beginning of the washout, by a transient enhancement of the bursting activity. This consisted of an increase in AP frequency within bursts induced by WM stimulation and intracellular depolarizing current injection (Figs. 7, 8, and 10).

**EFFECTS OF TETRODOKIN AND CADMIUM ON BURSTING CELLS.** The effects of the Na$^+$ blocker tetrodotoxin (TTX) were studied to determine the possible involvement of a Na$^+$ component in bursting patterns. APs evoked in SB (n = 2) and IB neurons (n = 2) by WM stimulation and depolarizing current injection were reversibly blocked (after 3-5 min) by adding 0.5 $\mu$M of TTX to the perfusion medium, indicating that these APs are Na$^+$ dependent and TTX sensitive (Fig. 11). After the addition of Cd$^{2+}$ (0.5 $\mu$M) in the presence of TTX in the perfusion medium, the intracellular depolarizing current evoked a depolarizing transient wave of $\sim$2-4 mV in amplitude and 100 ms in duration (Fig. 11). These results indicate that the Ca$^{2+}$-dependent potentials and the underlying Ca$^{2+}$-independent slow depolarization are TTX resistant.

**EFFECTS OF A CA$^{2+}$ CHELATOR ON BURSTING NEURONS.** The intracellular effects of the calcium chelator EGTA were tested in three bursting neurons. In two SB neurons, EGTA (500 mM in the micropipette) prolonged the depolarizing wave underlying bursts and favored the appearance of additional APs (Fig. 12). In one IB neuron, EGTA (250 mM) provoked a slight increase in the PDS amplitude (5-10%) similar to that observed by Witte et al. (1987) in the rat penicillin focus. In the same neuron, EGTA enhanced the bursting induced by depolarizing current injection. All these effects appeared 30-40 min after impalement and were observed during $\geq$1 h. These modifications probably did not result from unspecific effects caused by intracellular depolarizing currents (0.1-1.2 nA) were injected. EGTA diffusion had no detectable effect on the resting membrane potential or membrane impedance.

**EFFECTS OF CALCIUM ANTAGONISTS ON NONBURSTING NEURONS.** EPSPs evoked in NB cells were not affected by Ca$^{2+}$ antagonists (Verapamil, n = 3; Mg$^{2+}$, n = 3; or Cd$^{2+}$, n = 2) at concentrations able to suppress the bursting patterns. Peak latencies, as well as rise and fall of EPSPs, remained constant before, during, and after exposure to Ca$^{2+}$ antagonists. Moreover, the resting membrane potential and the input resistance were unchanged. These results, together with the lack of effect of Ca$^{2+}$ antagonists on EPSPs in bursting cells, indicate that the observed blocking action of

---

**FIG. 8.** Increased bursting in an IB neuron after offset of verapamil application (10 $\mu$M). A: PDS triggered by suprathreshold WM stimulation (●). EPSP to a subthreshold stimulation is superimposed in the top. B: intrinsic response triggered by suprathreshold WM stimulation verapamil (third traces in A and B) ($E_m = -75$ mV).
BURST GENERATION AFTER GABA WITHDRAWAL

Ca²⁺ antagonists on intrinsic burst genesis results mainly from a specific reduction of transmembrane Ca²⁺ currents.

DISCUSSION

The use of both in vivo and in vitro experimental preparations enabled identification of excitatory processes leading to epileptic manifestations of GWS in rats. In vitro experiments provided a description of IB and SB properties of neurons recorded in neocortical slices obtained from rats presenting GWS. Results demonstrate the cooperation of at least three different currents in the generation of PDSs: two synaptic currents, the classical EPSP and an NMDA receptor-mediated component, and a voltage-dependent Ca²⁺ current. In addition, experiments performed in living rats enabled the evaluation of the role of Ca²⁺ currents and
NMRA receptor-related currents in both the established GWS epileptic manifestations and epileptogenesis, i.e., processes that develop before interruption of GABA infusion and lead to GWS.

**Activation of NMRA receptors during synaptic responses**

The present data demonstrate the influence of excitatory amino acid-like transmitters active on NMRA receptors during GWS. At the doses used, APV and APH did not affect EPSPs underlying PDSs in IB and SB cells or EPSPs triggered in NB cells, although they were able to reduce PDSs. Therefore the waveform underlying PDSs in these neurons appears to be the combination of an early depolarization insensitive to low doses of APV or APH and of a later component that is affected by NMRA receptor antagonists. Excitatory amino acids may be responsible for the later component of PDS, as similarly shown for epileptiform bursting in the pyramidal hippocampal neurons (Dingledine et al. 1986; Herron et al. 1985) and for NMRA-induced depolarizations in neocortical neurons (Sutor and Hablitz 1989).

In the present in vivo experiments, phencyclidine administration that antagonizes NMRA receptor activation was unable to suppress epileptic manifestations of GWS. Moreover, PDSs of IB and SB neurons recorded in vitro were not entirely abolished by APV or APH. These antagonists were able to suppress PDSs induced in NB neurons after removal of extra-cellular Mg²⁺, as also demonstrated by Mody et al. (1987) in hippocampal slices. Therefore PDSs occurring during GWS cannot be exclusively related to activation of NMRA receptors or modification of the voltage-dependent Mg²⁺ control of NMRA current. We suggest a contribution in PDSs of a third component that differs from EPSPs and NMRA currents, probably a voltage-dependent Ca²⁺ current.

**Calcium currents are responsible for bursting patterns**

In contrast to APV or APH, organic and inorganic Ca²⁺ blockers at the doses used were able to entirely abolish intrinsic bursts in IB neurons, strongly depress PDSs in both IB and SB neurons, and suppress bursts of APs superimposed on these PDSs without affecting EPSPs triggered in the same neurons. Therefore the effects of Ca²⁺ blockers are not related to suppression of Ca²⁺-dependent release of transmitters. These findings agree with the observations of Louvel et al. (1986), who showed that calcium antagonists at low dosages have only small effects on Ca²⁺ entry into presynaptic structures. Moreover, Higashi et al. (1990) demonstrated that the concentration of organic Ca²⁺ blockers necessary to depress EPSP amplitude was 2–10 times higher than that necessary to shorten the Ca²⁺ spike. Thus, during GWS, synaptic stimulation elicits pyramidal IB and SB neurons an EPSP that is relatively resistant to Ca²⁺ blockers, an NMRA receptor-mediated component, and a voltage-dependent Ca²⁺ current resistant to TTX. It is possible that this voltage-dependent, current together with non-NMRA receptor activation (Asher and Nowak 1988), contributes to the depolarizing step that is required to overcome blockade of NMRA receptors by extracellular Mg²⁺ ions.

The Ca²⁺-dependent component of bursting patterns during GWS could be related to activation of L channels, as suggested by similar patterns of intrinsic bursts (Johnston et al. 1980; Wong and Prince 1978; 1979) or PDSs (Lux and Heinemann 1983; Schwartzkroin and Prince 1980; Traub and Llinas 1979) recorded in hippocampal pyramidal cells. Involvement of L channels would explain the high sensitivity to Ca²⁺ blockers and the regulatory effect of intracellular Ca²⁺ ions sensitive to the injection of EGTA.

Evidence for a fourth component was obtained by analyzing the responses of IB neurons to a depolarizing current injection in the presence of Ca²⁺ blockers. This component is a TTX-resistant transient depolarization that is reminiscent of either a transient (T or N) Ca²⁺ current relatively insensitive to Ca²⁺ blockers (Fox et al. 1987) or a slowly activating, noninactivating (possibly K⁺) current, such as
the I_m current (Brown 1988). Both low-threshold inactivating (T and N types) and high-threshold noninactivating (L type) currents have been documented in voltage-clamp recordings of cortical pyramidal cells with the use of K^+ blockers (Connors et al. 1982; Franz et al. 1986; Stafstrom et al. 1985). We therefore conclude that, although primarily synaptic in nature, PDSs involve voltage-dependent Ca^{2+} conductances during GWS. Further experiments are required to identify these voltage-dependent currents likely underlying PDS.

Mixed contribution of synaptic and voltage-dependent processes to GWS

Voltage-dependent Ca^{2+} current in IB cells appears to be a major electrophysiological correlate of GWS at the neuronal level. However, in vivo administration of flunarizine has virtually no effect on GWS, although it is relatively active on verapamil-sensitive Ca^{2+} currents of neocortical neurons recorded in vitro (Bingmann et al. 1988). In fact, epileptic activities during GWS involve both synaptic and intrinsic components. In living animals, EPSPs relatively resistant to NMDA antagonists are probably responsible for elicitation of PDSs that are therefore insensitive to flunarizine. The combination of voltage-dependent currents and synaptic (e.g., EPSP or NMDA) components may explain why PDSs are relatively resistant to low doses of Ca^{2+} blockers in vivo.

Results obtained in in vivo experiments suggest that GWS may constitute a good model of status epilepticus, allowing the pharmacological analysis of anticonvulsant drug action (Woodbury 1983). In living rats, GABA_{\alpha} agonists (clonazepam, diazepam), NMDA antagonists [MK 801, APH, Menini et al. unpublished observations], and phenytoin (see above) were unable to suppress seizures when administered after interruption of GABA infusion. We consider that failure of proper pharmacological treatment is a consequence of the complex generation of PDSs as long as GABA is applied (i.e., for periods of several hours). In the presence of GABA, NMDA-related currents are not activated because of the voltage-dependent blockade by Mg^{2+} ions (Ascher and Nowak 1988; MacDermott et al. 1986). Thus the antiepileptogenic property of NMDA antagonists may probably depend on an action outside the infusion site. Therefore connections between the infusion site and the noninfused areas must be critical for epileptogenesis. Anatomic observations indicate that during GWS the "abnormal" (IB and SB) pyramidal neurons are located within 0.7-1.2 mm of the infusion site. Neurons located at larger distances are all NB neurons and are probably not directly affected by the GABA infusion.

A downregulation of GABA synthesis by chronic GABA infusion could be involved in the focal appearance of epileptic activities on GABA withdrawal. Glutamic acid decarboxylase immunoreactive nerve terminals within the focus appear markedly decreased during the first hours after epileptic discharges appearance, whereas no change was observed in controls (saline infused rats). This result indicates a decrease in GABA synthesis and release due to the local prolonged GABA infusion (L. Wiklund, personal communication). GWS would therefore critically depend on the unbalanced synaptic relationship between neurons in infused and noninfused zones.

We report here the first evidence for an epileptogenic (instead of antiepileptic) consequence of Ca^{2+} current blockade due to GABA infusion. We have consistently observed that the offset of organic and inorganic Ca^{2+} blocker application (but not of NMDA antagonists) in vitro was followed by an exaggeration of bursting patterns in IB and SB neurons. As already mentioned, GABA infusion maintains the neuronal membrane at a hyperpolarizing level, below the threshold for elicitation of voltage-dependent Ca^{2+} currents. It is thus expected that prolonged GABA infusion reduces most Ca^{2+} currents induced at depolarized membrane potentials in pyramidal cells. This decrease in intracellular Ca^{2+} concentration may contribute to the appearance, on GABA withdrawal, of bursting patterns. In the same way, a decrease in intracellular Ca^{2+} correlates to an increase of excitability of bursting neurons after intracellular EGTA diffusion. Thus our working hypothesis is that a reduction of Ca^{2+} may contribute to GWS.

Moreover, the GABA intracortical infusion may have induced a long-lasting change of Ca^{2+} channels and downregulation of GABA_{\alpha} receptors, as well as a change in their state of phosphorylation or conformation (Byerly and Hagiwara 1988; Stelzer et al. 1988). These changes seem required to enhance bursting after reduction of Ca^{2+} currents or intracellular Ca^{2+}. Exaggeration of bursting patterns after offset of Ca^{2+} blockade was seen only in IB or SB neurons. In no case was a bursting pattern generated de novo after offset of blockers in NB cells. Thus the present study suggests that processes leading to seizures result from the combination of a GABA action and a reduction of Ca^{2+} influx at the site of GABA infusion.

In conclusion, the present study provides evidence for a large Ca^{2+} influx through voltage-dependent channels in cortical bursting neurons during GWS as a possible consequence of combined GABA action and lowered Ca^{2+} influx during previous GABA infusion. After GABA interruption, large Ca^{2+} influxes develop at the site of infusion and suppress postsynaptic sensitivity to GABA_{\alpha} agonists (Deisz
and Lux 1985; Stelzer et al. 1988), as we have already demonstrated in vivo and in vitro (Champagnat et al. 1990; Silva-Barrat et al. 1989). All these factors can contribute to the appearance of an epileptic focus containing neurons that present intrinsic bursts and synchronically driven bursts. We propose that in GWS, as in human epilepsia partialis continua (Engel et al. 1983), cerebral dysfunction and subsequent abnormal relationships between neurons within the focus and the neighborhood are critical for the development of epileptic manifestations.

The authors acknowledge J. Louvel for the gift of Verapamil, G. Levesque, G. Ghilini, and J. P. Boullot for skillful assistance, and S. Orsoni for aid in manuscript preparation.

This work was supported by grants from La Fondation de France and La Fondation pour la Recherche Médicale.

Present address of S. Araneda: Institut des Neurosciences, Université Pierre et Marie Curie. 7 Quai St Bernard, 75252 Paris Cedex 05, France.

Address for reprint requests: C. Silva-Barrat, Laboratoire de Physiologie Neuroveine, C.N.R.S. 91198 Gif-sur-Yvette Cedex, France.

Received 6 May 1991; accepted in final form 14 November 1991.

REFERENCES


LUX, H. D. AND HEINEMANN, U. Consequences of calcium-electrogenesis...


