Pharmacology Biochemistry and Behavior, Vol. 65, No. 4, pp. 577–583, 2000

0091-3057/00/$–see front matter

ELSEVIER

PII S0091-3057(99)00250-6

Ricinine-Elicited Seizures: A Novel Chemical Model of Convulsive Seizures

ANETE C. FERRAZ,* LUIZ FERNANDO PEREIRA,† RAFAELA L. RIBEIRO,* CLAUDIA WOLFMAN,‡ JORGE H. MEDINA,‡ FÚLVIO A. SCORZA,§ NEIDE F. SANTOS,§ ESPER A. CAVALHEIRO§ AND CLAUDIO DA CUNHA*

**Laboratório de Fisiologia e Farmacologia do SNC, Departamento de Fisiologia e Dep. Farmacologia, Univ. Fed. Paraná, C.P. 19.031, 81.531-990 Curitiba, PR, Brazil; †Departamento de Bioquímica, Univ. Fed. Paraná, Curitiba, PR, Brazil; ‡Instituto de Biologia Celular, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina; and §Laboratório de Neurologia Experimental, Universidade Federal de São Paulo (UNIFESP-EPM), São Paulo, SP, Brazil*

Received 22 January 1999; Revised 19 July 1999; Accepted 6 August 1999

FERRAZ, A. C., L. FERNANDO PEREIRA, R. L. RIBEIRO, C. WOLFMAN, J. H. MEDINA, F. A. SCORZA, N. F. SANTOS, E. A. CAVALHEIRO AND C. DA CUNHA. *Ricinine-elicited seizures: A novel chemical model of convulsive seizures.* PHARMACOL BIOCHEM BEHAV **65**(4) 577–583, 2000.—The present investigation introduces ricinine-elicited seizures as a novel chemical model of convulsive seizure. Ricinine, a neutral alkaloid obtained from the plant *Ricinus communis*, induces seizures when administered to mice at doses higher than 20 mg/kg. Animals presenting seizures showed a marked preconvulsive phase followed by short duration hind limb myoclonus, respiratory spasms, and death. The lethal nature of ricinine seizures is also pointed out as a good model to study the events causing death in clonic seizures, particularly those related to respiratory spasms, which are also observed in some types of human epilepsy. The behavioral signs of ricinine-elicited seizures are accompanied by electrographic alterations more evident during the preconvulsive phase in the cerebral cortex and more intense during the ictal phase both in the cortex and in the hippocampus. The ricinine-elicited seizures may be inhibited by diazepam but not by phenobarbital, phenytoin, or ethosuximide. Micromolar concentrations of ricinine cause a small decrease in the binding of [^{3}H]-flunitrazepam to cerebral cortex membranes, but do not alter the binding of other radioligands to AMPA, 5-HT$_{1A}$, muscarinic, and α_1-adrenergic receptors. Although ricinine presents a cyanide radical, only higher doses of ricinine (4 mM) caused a small impairment of mitochondrial respiration. These results suggest that the mechanism of action of ricinine probably involves the benzodiazepine site in the GABA$_A$ receptor. This may represent a new mechanism of drug-elicited seizures that may contribute to a better understanding of epilepsy and to new therapeutic approaches to this disease. © 2000 Elsevier Science Inc.

Ricinus communis Chemical model Seizures EEG Mechanisms

RICININE is a neutral alkaloid isolated from the plant *Ricinus communis.* Mice receiving a high dose of ricinine present clonic seizures and other signs of central nervous system (CNS) stimulation (10). At lower doses, ricinine, like many other CNS-stimulants, improves memory retention. Most of the drugs that improve memory at lower doses (17,18,20) elicit seizures when administered at higher doses (5,13). The characteristics of the seizures, the mechanisms by which they are induced by the drug, and the properties of anticonvulsants that prevent them have been used as chemical models of epilepsy (5).

The mechanism of action of ricinine is not known. Farah et al. (9) suggested that ricinine inhibits the mitochondrial respiratory chain. There are many other mechanisms that theoretically may explain the CNS-stimulant effects of ricinine, including an interaction with neuroreceptors known to be affected by most of the known convulsant drugs.

In the present investigation we studied the electrophysio-

Requests for reprints should be addressed to Dr. Cláudio Da Cunha, Laboratório de Fisiologia e Farmacologia do SNC, Dep. Farmacologia, Univ. Fed. Paraná, C.P. 19.031, 81.531-990 Curitiba, PR, Brazil.

logical, behavioral, and neurochemical characteristics of ricinine as a convulsant. The mechanism of action of the drug was studied by testing its interference with the binding of radioligands to some neuroreceptors and with mitochondrial respiration, and by studying the protective effect of anticonvulsants on ricinine-elicited seizures.

METHOD

Animals

The behavioral and electroencephalographic experiments were performed on 290 adult male Swiss mice (2.5 months, 20–30 g) from our own breeding stock housed in groups of 15 in Plexiglas boxes (60 × 25 × 25 cm). Biochemical assays were performed on cerebral cortex membranes or isolated liver mitochondria from 19 adult male Wistar rats (2.5 months, 220–300 g) from our own breeding stock housed in groups of six in similar home-cages. The animals were maintained at 22 ± 2°C on a 12 L:12 D period (lights on 0700 h) with food and water available ad lib. Behavioral experiments using a large number of animals could be performed with mice because pilot experiments showed that the general effect of ricinine was observed both in mice and in rats. Electroencephalographic records were performed with mice to compare the electrographic records with behavior. Biochemical essays were done with rat tissues because of the larger size of the rat organs compared to mice, and also because the techniques used in our laboratories were standardized for rat tissues. Furthermore, the basic biochemical processes studied in general do not differ between rats and mice.

Drugs and Injection Procedures

The tests were always carried out between 0700 and 1200 h. All drugs and plant extracts were administered in a volume of 0.01 ml/kg. Ricinine was purified from the pericarps of *R. communis*, as described elsewhere (10). Ricinine was prepared in a mixture of corn oil:water (1:1), with a drop of Tween 80 per ml of water, and was administered subcutaneuosly (SC). The range of doses is described below for each set of experiments. Diazepam was obtained from Hoffmann–La Roche (USA). Phenobarbital was purchased from Polfa (Pozman, Poland). Phenytoin was acquired from Desitin (Hamburg, Germany). Ethosuximide was obtained from Sigma (St. Louis, MO). All drugs were dissolved in a 3% solution of Tween 80 (Sigma) and 0.9% NaCl. Binding assays were performed with the following radioligands purchased from NEN-Dupont (Wilmington, DE): [^{3}H]-Flunitrazepam, 87.0 Ci/mmol; [^{3}H]-muscimol, 19.5 Ci/mmol; [^{3}H]-AMPA, 53.0 Ci/mmol; [^{3}H]-8 OH-DPAT (8-hydroxy-2-(di-n-propilamino)tetralin), 154.3 Ci/mmol; [^{3}H]-zolpidem, 50.8 Ci/mmol; [^{3}H]-N-methyl scopolamine, 84.0 Ci/mmol; and [^{3}H]-prazosin, 72.0 Ci/mmol.

Experiment 1: EEG Recordings of Ricinine-Elicited Seizures

For EEG recordings (Beckman model RM polygraph, time constant 0.03 s, high cutoff filter 15 Hz) bipolar twisted electrodes (tip diameter 100 μm, interelectrode distance 500 μm) were stereotaxically positioned in the dorsal hippocampus according to the coordinates of the Montemurro and Dukelow atlas (16): (AP) −1.5 mm from bregma; (ML) +2.4 mm from midline; (DV) −1.3 mm from skull. For surgeries the animals were anesthetized with a mixture of 40 mg/kg thionembutal and 170 mg chloral hydrate. Surface recordings were made from screws positioned bilaterally over the sensorimotor cortex. An additional screw placed over the frontal sinus served as a reference electrode. Three days after surgery, control recordings were taken for approximately 15 min and 10, 20, 30, or 40 mg/kg ricinine were administered SC to groups of 10 mice each. The occurrence of exophthalmus, decreased locomotor activity, tremor, jumping, apnea, clonic hind limb clonic seizures, death, and the corresponding time of each occurrence were observed at the same time as the EEG recordings. A pilot experiment showed that the vehicle did not alter the behavioral and electrographic observations made in mice. At the beginning of this experiment a control record was obtained from each animal before it received the proper dose of ricinine.

The electrographic record presented as control was obtained from an animal before the injection of any drug.

Experiment 2: Effect of Anticonvulsant Drugs on Ricinine-Elicited Seizures

Two hundred and fifty additional mice were equally divided into five groups that received IP saline, 10 mg/kg diazepam, 20 mg/kg phenobarbital, 50 mg/kg phenytoin, or 400 mg/kg ethosuximide. Thirty minutes later, each group were subset into five groups of 10 animals each that received SC vehicle, 10, 20, 30, or 40 mg/kg ricinine. These animals were observed for 4 h and the number of animals that presented exophathalmos, tremors, jumping, hind limb clonic seizures, or died was recorded.

Experiment 3: Effect of Ricinine on Mitochondrial Respiration

The effect of ricinine on mitochondrial respiration was studied in rat liver mitochondria prepared according to the procedure of Voss et al. (24) with minor modifications. The livers for the mitochondrial preparations were obtained from a total of nine rats sacrificed by decapitation. The mitochondrial pellet was washed twice and suspended in 250 mM mannitol, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 10 mM HEPES-KOH (pH 7.2), and 0.1% bovine serum albumin (BSA). Oxygen consumption was measured polarographically with a Clark electrode (8) connected to a Gilson recorder. The reactions were carried out in a 1.5-ml water-jacketed closed chamber with magnetic stirring at 30°C in a medium containing the purified mitochondria (1.75 mg protein determined by the method of Lowry et al. (14), 125 mM mannitol, 65 mM KCl, 0.1 mM EGTA, 10 mM HEPES-KOH, 0.1% BSA, 5 mM phosphate buffer (pH 7.2), and 5 mM α-ketoglutarate. Respiratory control (RC), state III, state IV and ADP/O ratio were calculated according to Estabrook (8).

Experiment 4: Effect of Ricinine on the Binding of Radioligands to Neuroreceptors

The binding assays were performed on rat cerebral cortex membranes obtained from a total of 10 rats sacrificed by decapitation. The cerebral cortex was dissected from the brains on ice. All the subsequent procedures were performed at less than 4°C. The tissues were homogenized in 10 vol of 0.32 M sucrose and centrifuged for 10 min at 1000 × *g*. The supernatant was separated, and the pellet was rehomogenized and again centrifuged as above. The two supernatants were pooled and centrifuged at 40,000 × *g* for 30 min to yield a pel-

let corresponding to a crude synaptosomal membrane fraction. These membranes were homogenized in 20 vol of the appropriate buffer, centrifuged at 40,000 × *g* for 30 min, and stored at −70°C until utilized.

Central type benzodiazepine receptors were measured using [^{3}H]-flunitrazepam binding (0.15 nM) in 25 mM Tris-HCl buffer, pH 7.4. ω_1-Benzodiazepine receptors were measured using [^{3}H]-zolpidem (1 nM) in 50 mM Tris-HCl buffer, pH 7.4; glutamate AMPA receptors were measured using [^{3}H]-AMPA (10 nM) in 50 mM Tris-HCl buffer, pH 7.4; $GABA_A$ receptors were measured using [^{3}H]-muscimol (2nM) in 25 mM Tris-HCl buffer, pH 7.4; 5-HT_{1A} receptors were measured using [^{3}H]-8 OH-DPAT (0.5 nM) in 50 mM Tris-HCl buffer, pH 7.4; muscarinic receptors were measured using [^{3}H]-*N*-methyl-scopolamine (150 pM) in 50 mM phosphate-HCl buffer, pH 7.4; α_1-adrenergic receptors were measured using [^{3}H]-prazosin (1 nM) in 50 mM Tris-HCl buffer, pH 7.4. In all cases the concentration of the radioligand was near the K_D. For each assay, triplicate membrane samples containing 0.1 mg protein determined by the method of Lowry et al. (14) were suspended in 1 ml of the reaction buffer. Incubation was carried out for 60 min at 4°C, except for [^{3}H]-8 OH-DPAT binding, for which the preparation was incubated at 25°C. Nonspecific binding amounted to 10–20% of the total, and was determined in a parallel incubation in the presence of saturating concentrations of a nonradioactive ligand (3 μM flunitrazepam, 50 μM zolpidem, 1 mM glutamate, 100 μM GABA, 50 μM 5-HT, 25 μM atropine or 200 μM prazosin, respectively). Binding was stopped by rapid filtration through GF/B filters with 3 washes of 6 ml each of the incubation buffer. The filters were dried and transferred to vials containing a scintillation cocktail (2.5% diphenyloxazole-xylene) and radioactivity was measured with 40% efficiency.

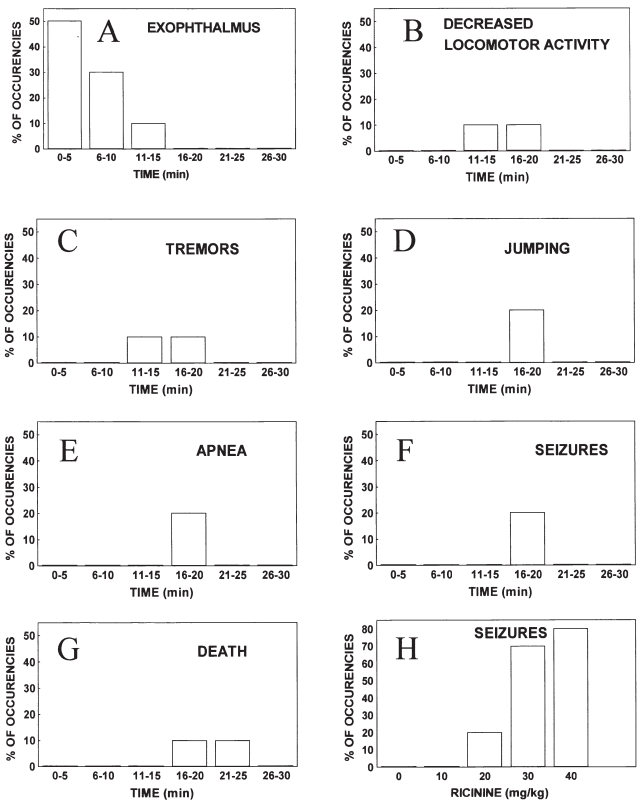

FIG. 1. Typical time-dependent and dose-dependent behavioral characteristics of seizures elicited by ricinine in mice. In histograms A–G, the columns represent the percentage of animals that presented each characteristic behavior or death after receiving 20 mg/kg ricinine. Histogram H represents dose–effect data about animals presenting clonic seizures up to 30 min after receiving ricinine.

Statistical Analysis

Data from Experiment 2 were analyzed by multiple linear regression comparing differences in the slopes and differences in the axis intercepts (GraphPad Prizma software). Data from Experiments 3 and 4 were analyzed by one-way ANOVA followed by the post-hoc Duncan test. Pearson's correlation coefficient for each group of data from Experiment 3 was calculated and was always <40% (Statistica software).

RESULTS

Animals that received 10 mg/kg ricinine did not present behavioral or electroencephalographic signs of seizure. Some animals receiving 20–40 mg/kg ricinine presented characteristic seizures that did not differ in behavioral or electrographic pattern or even in the mean onset latency, regardless of the ricinine dose. The percentage of animals that

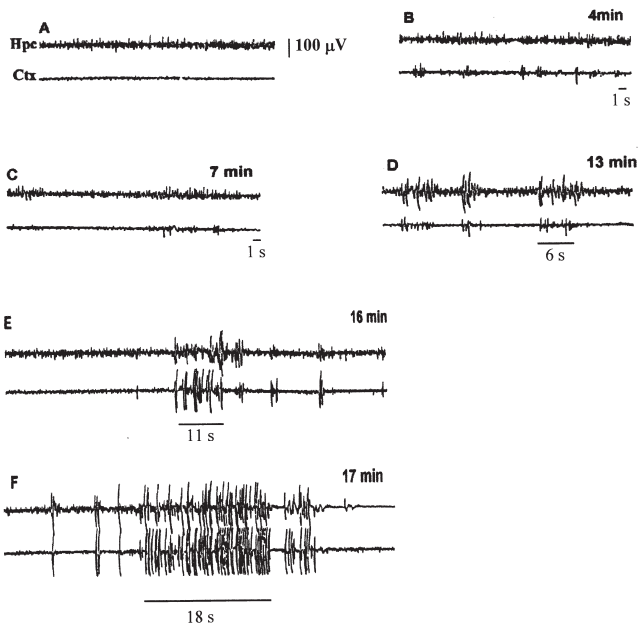

FIG. 2. Typical time-dependent electroencephalographic characteristics of seizures elicited by 20 mg/kg ricinine in mice. The recordings were obtained from animals chronically implanted with bipolar twisted electrodes prior to (A) control, and after receiving 20 mg/kg ricinine (B–F). Deep recordings were obtained from hippocampal-implanted electrodes and superficial recordings from cortical bilateral sensorimotor screws. The electroencephalographic recordings and the behavioral observations (Fig. 1) were obtained from the same animals.

TABLE 1
EFFECT OF ANTICONVULSANTS ON RICININE SEIZURE

Observation	Ricinine ED_{50} (mg/kg)				
	Control	Diazepam (10 mg/kg)	Phenobarbital (20 mg/kg)	Phenytoin (50 mg/kg)	Ethosuximide (400 mg/kg)
Clonic Seizure	27.5	56.2*	27.5	26.9	30.2
Tremors	29.5	79.4‡	28.2	16.6‡	25.1
Jumpings	29.5	173.7†	28.2	29.5	19.0*
Apnea	33.8	56.2*	28.2	21.8*	21.8*
Exophthalmus	13.4	32.3‡	41.7‡	10.0	14.4
Reduced locomotor Activity	28.8	41.7‡	26.9	13.5‡	28.8

ED_{50} was calculated by multiple linear regression (log × probit plot) from data obtained from animals that receive 10–40 mg/kg ricinine alone (control) or 30 min after receiving an anticonvulsant drug.
*$p \leq 0.05$; †$p \leq 0.01$; ‡$p \leq 0.001$ compared to control.

presented seizures increased with the dose as can be seen in Fig. 1H. Figure 1(A–G) and Fig. 2 summarize the typical behavioral and electrographic occurrences after ricinine administration. The electrographic recordings obtained for other animals presenting seizures were closely similar, except for the onset of the seizure signs ranging from 3 to 12 min. As showed in Fig. 2, 4 min after receiving ricinine, fast activity of low voltage isolated spikes was observed in the cerebral cortex. After 7 min, these cortical spikes were accompanied by synchronic hippocampal low voltage-increased activity, of longer duration than the cortical spikes. Concomitant with these electrographic alterations the animals presented prominent exophthalmus (see Fig. 1A). Thirteen minutes after ricinine administration, the isolated low voltage cortical bursts became wider, and the synchronic hippocampal activity increased in amplitude, being composed of both low- and high-voltage spikes. At that time, the burst of spikes lasted typically 2–6 s, and occurred at 0.1–0.3 Hz. The animals observed during this period were motionless and started to present generalized tremors that persisted in the subsequent phase. Between 16–17 min the cortical and hippocampal activity progressed from short periods (4–11 s) of high-voltage, high-frequency spikes to fully developed ictal activity that occurred during short periods of time (18 s), and were intercalated with short (1–2 s) silent activity. The corresponding behavior observed during this period progressed from respiratory distress to a series of myoclonic jerks that rapidly evolved to a generalized tonic–clonic seizure with respiratory failure followed by death of the animal.

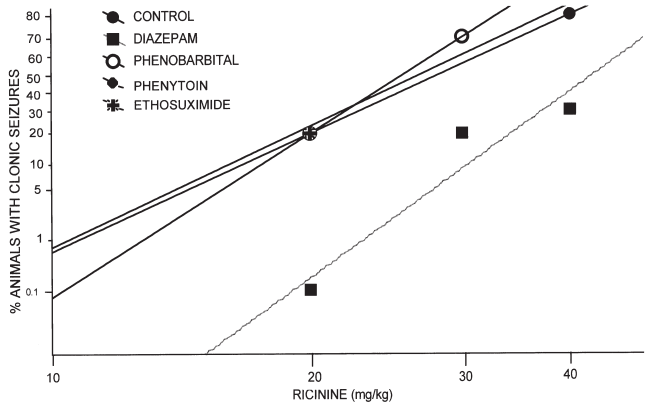

FIG. 3. Effect of anticonvulsants on ricinine-elicited seizures. Groups of 10 animals each received diazepam (10 mg/kg), phenobarbital (20 mg/kg), phenytoin (50 mg/kg), or ethosuximide (400 mg/kg), followed 30 min later by vehicle, or 10, 20, 30, or 40 mg/kg ricinine. The points represent the percentage of animals that presented seizures in a log(dose) × probit plot. Multiple linear regression analysis showed a significant difference between y-intercepts ($p \leq 0.01$) and no significant difference in the slopes of the curves ($p \geq 0.47$).

As can be seen in Table 1 and in Figure 3, diazepam increased the ED_{50} of ricinine to elicit clonic seizures, $F(4, 14) = 5.52$, $p \leq 0.01$, and the other behavioral characteristics of the ricinine seizure: tremors, $F(4, 14) = 9.31$, $p \leq 0.001$; jumping, $F(4, 14) = 9.56$, $p \leq 0.001$; apnea, $F(4, 14) = 3.11$, $p \leq 0.05$; exophthalmus, $F(4, 14) = 12.90$, $p \leq 0.001$, and reduction of locomotor activity, $F(4, 14) = 16.32$, $p \leq 0.001$. None of the other anticonvulsants protected the animals against ricinine-induced clonic seizures ($p \geq 0.2$), and some of them even exacerbated some characteristics of ricinine seizures other than clonic convulsions (see Table 1). Further experiments (not shown) showed that phenobarbital did not protect seizures induced by 30 mg/kg ricinine even when administered at a dose of 60 mg/kg.

The data presented in Table 2 show that 30 or 100 μM ricinine caused a small but significant decrease in the binding of [^{3}H]-flunitrazepam to the central benzodiazepine receptors of rat cerebral cortex membranes [one-way ANOVA, $F(2, 6) = 121.08$, $p \leq 0.001$, followed by the Duncan test, $p \leq 0.001$]. This minor effect seems to be specific because the same concentrations of ricinine did not alter the binding of radioligands to the other neuroreceptors studied ($p > 0.2$), including the ω_1-benzodiazepine receptor agonist, [^{3}H]-zolpidem.

As presented in Table 3, 4 mM ricinine increased the rate of state IV [one-way ANOVA, $F(2, 13) = 8.57$, $p \leq 0.01$] of mitochondrial respiration and decreased the respiratory control rate (RC), $F(2, 13) = 5.37$, $p \leq 0.05$, without affecting the state III rate, $F(2, 13) = 2.12$, $p = 0.16$, or the ADP/O ratio, $F(2, 13) = 3.39$, $p = 0.07$. Individual differences between groups are presented in Table 3.

DISCUSSION

The present results introduce ricinine as a new chemical model of convulsive seizures that may contribute to the eluci-

TABLE 2

EFFECT OF RICININE ON THE BINDING OF RADIOLIGANDS TO NEURORECEPTORS

Neuroreceptor	Radioligand	% Binding		
		Control	30 μM Ricinine	100 μM Ricinine
$GABA_A$	[^{3}H]-muscimol	100 ± 3	110 ± 3	86 ± 12
AMPA	[^{3}H]-AMPA	100 ± 13	138 ± 5	115 ± 21
$5\text{-}HT_{1A}$	[^{3}H]-8 OH-DPAT	100 ± 9	100 ± 4	103 ± 1
Central Benzodiazepine Receptor	[^{3}H}-flunitrazepam	100 ± 1	83 ± 1*	71 ± 1*
ω_1-Benzodiazepine Receptor	[^{3}H]-zolpidem	100 ± 3	91 ± 3	93 ± 1
Muscarinic	[^{3}H]-*N*-methyl scopolamine	100 ± 3	96 ± 2	96 ± 1
α_1-Adrenergic	[^{3}H]-prazosin	100 ± 3	81 ± 14	97 ± 6

Ricinine was incubated with rat cerebral cortex membranes. The results represent mean percentage ± SEM. of specific [^{3}H]-radioligand binding considering the binding in the absence of ricinine as a control group.

*$p \leq 0.001$, compared to control group, Duncan test after one-way ANOVA.

dation of new mechanisms involved in epilepsy and to new therapeutic approaches to this disease.

The most characteristic signs observed during ricinine seizures initiate after a few minutes with prominent exophthalmus, decreased locomotor activity, muscular tremors, and jumping, followed by respiratory spasms and major hind limb myoclonus. All of these behavioral signs matched the electroencephalographic activity, which started with small spikes in the cerebral cortex and hippocampus, and increased to larger and massive discharges observed during ictal activity. All animals that presented clonic seizures died a few minutes later, probably as a consequence of a respiratory arrest because the hearts of these animals continued beating after they stopped breathing. The literature reports the occurrence of apnea and respiratory spasms associated with some kinds of human epilepsy seizures like the sleep apnea syndrome and the pediatric congenital bilateral perisylvian syndrome (6,11). Thus, ricinine-elicited seizures may be a putative model of seizures matching this symptom.

Compared to the other chemical models of seizures, the ricinine model seems to be quite different. Drugs acting as antagonists of the Cl^- ionophore–$GABA_A$ complex, like bicuculline, picrotoxin, and pentylenetetrazole, normally induce reversible and more prolonged seizures, a fact that does not occur with ricinine-elicited seizures that always cause animal death. Furthermore, seizures elicited by those convulsants are more generalized, encompassing oral-facial, and fore- and hind limb clonic convulsions, intercalated with small clonic–tonic seizures (3,15,21), while ricinine-elicited seizures are shorter, and mainly restricted to hind limb movements. Another marked difference of ricinine-elicited seizures compared to these drugs is the respiratory spasms elicited by it, which seem to be the main cause of ricinine lethality. The glycine antagonist, strychnine, like ricinine, induces seizures in which the animals die during a respiratory arrest (4) but, in this case, the effect is due to a tonic seizure that is not observed in ricinine-elicited seizures. Compared to the seizures elicited by glutamate receptor agonists such as kainic and ibotenic acids, ricinine seizures do not have a silent period followed by recurrent seizures (2). The recurrent seizures induced by intracerebral or systemic administration of higher doses of cholinomimetics like pilocarpine have being suggested as chemical models of epilepsy (22). The electroencephalographic activity observed in the cerebral cortex and in the hippocampus of mice during ricinine-elicited seizures is different from those observed in pilocarpine-elicited seizures (23): pilocarpine seizures initiate with increased activity in the hippocampus, while ricinine induces an increase in electrographic activity that is more marked in the cerebral cortex in the first stage, being more evident in the hippocampus only a few minutes later; pilocarpine-elicited seizures present larger bursts of high-voltage, high-amplitude spikes, normally lasting more than 25 s, while ricinine-elicited seizure present narrower high-voltage, high-amplitude bursts lasting less than 20 s and normally intercalated with short (1–2 s) silent periods; ricinine seizures last no more than 15 min, while pilocarpine-elicited seizures may last hours. The main differences between two models are that the latter induces recurrent seizures while ricinine induces lethal seizures. In this way, as pointed out above, the ricinine model may be a tool to study the events in a clonic seizure that cause mortality. Furthermore, it may be one of the few animal models of seizures in which the ep-

TABLE 3

EFFECT OF RICININE ON THE MITOCHONDRIAL RESPIRATORY RATE

Group	(Nano-atoms of Oxygen Consumed × min^{-1} × mg $Protein^{-1}$)			
	State III	State IV	R.C.	ADP/O
Control	69.12 ± 3.30	15.23 ± 1.92	4.80 ± 0.53	2.27 ± 0.10
2 mM Ricinine	56.84 ± 2.06	15.03 ± 2.38	4.20 ± 0.64	2.81 ± 0.32
4 mM Ricinine	60.72 ± 5.22	23.68 ± 0.81*‡	2.80 ± 0.10*†	2.18 ± 0.03

The effect of ricinine on respiratory rate of state III, state IV, respiratory control (R.C.), and the ADP/O ratio were studied in rat liver mitochondrial preperations incubated in a medium containing α-ketoglutarate as substrate. Data represent the mean ± SEM of five to six determinations per group.

*$p \leq 0.01$, Duncan test after ANOVA, compared to control group.

†$p \leq 0.05$ compared to the 2 mM group, ‡$p \leq 0.01$ compared to the 2 mM group. Pearson's correlatoin coefficient was < 39%.

ileptogenic activity does not initiate in limbic structures but most possibly in a cortical area.

Among the anticonvulsants studied, only diazepam protected mice against ricinine seizures. Phenobarbital at the dose of 20 mg/kg normally protects mice against seizures elicited by all the other known convulsants (5, 19), but it did not protect the mice against ricinine-induced seizures even at the dose of 60 mg/kg. This is amazing since the anticonvulsant properties of barbiturates and benzodiazepines are explained by a similar mechanism of action, i.e. interaction with $GABA_A$ receptors. These results suggest that ricinine binds to a site of the $GABA_A$ receptor that is close to the site of the benzodiazepines but far from the site of barbiturates. It may also be explained by specificity for $GABA_A$ subtypes.

Our binding experiments showed that only concentrations in the μM range may cause a small decrease in [^{3}H]-flunitrazepam binding to cerebral cortex membranes, while the binding of the ω_1-benzodiazepine receptor radioligand, [^{3}H]-zolpidem, was not impaired at all. In addition, ricinine did not displace [^{3}H]-muscimol binding, indicating that this alkaloid does not interact with the GABA site of the $GABA_A$ receptor complex. A possible explanation to be explored in future experiments may be that ricinine could bind to specific subtypes of the GABA/BZ/Cl^- ionophore complex at a site close to the benzodiazepine receptor and that, at the same time, it decreases Cl^- flux and allosterically decreases the affinity of the receptor to benzodiazepines. The other binding results point against the participation of AMPA, 5-HT_{1A}, muscarinic, and α_1-adrenergic receptors in the ricinine mechanism. Moreover, the lack of a protective effect of the other tested anticonvulsants also decreases the possibility of involvement of voltage-dependent sodium channels, sodium-potassium ATPase, Ca^{2+}-calmodulin protein kinase, and T-type Ca^{2+} currents [see phenytoin and ethosuximide mechanisms in (19)]. Besides, our results showed that phenytoin and ethosuximide potentiated some of the ricinine preconvulsive behavioral signs.

Farah et al. (9) suggested that ricinine should elicit seizures that impair mitochondrial respiration. In theory, this would be possible because the nitrile group present in ricinine may resemble cyanide, a poison known to block the electron flow in cytochrome oxidase, a component of the mitochondrial respiratory chain (12). Neural mitochondrial failure would be similar to an anoxic insult affecting energy-dependent ionic pumps and the ability of neurons to maintain the resting membrane potential (7). As a consequence, neuron membranes depolarize, and may initiate epileptogenic activity. The results we present above show that ricinine may inhibit mitochondrial respiration by increasing state IV, but the concentration necessary to cause this effect is excessively high (see Table 3). It is highly improbable that mice receiving 20–40 mg/kg ricinine would concentrate it to 4 mM in the mitochondria of cerebral cortex neurons. Furthermore, if this were the ricinine mechanism of action, diazepam would be expected to protect animals of clonic seizures but not to protect them against death as it did.

Taken together, the present results introduce systemic administration of ricinine to mice as a novel chemical model of convulsive seizures. By its physiological characteristics it offers a good model to study lethal seizures, particularly those eliciting respiratory spasms. Furthermore, the results of our experiments suggest that the mechanism of action of ricinine is related to an unidentified site in the $GABA_A$ receptor, and point against the involvement of other neuroreceptor systems.

ACKNOWLEDGEMENTS

This research was supported by CNPq, LANBIO, and PRONEX. The authors are indebted to Miguelina Levi de Stein, Dr. Edgar Kornisiuk, and Dr. Haydee Viola for their participation in some binding experiments. The financial support of CNPq and PRONEX is also acknowledged.

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