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Antiepileptogenic properties of phenobarbital: behavior and neurochemical analysis

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Abstract

Chronic in vivo models of epilepsy provide a suitable strategy for quantifying epileptogenesis, as well as investigating neurochemical changes associated with neuronal plasticity that leads to seizuring conditions. The aim of this paper was to investigate antiepileptogenic properties of phenobarbital, focusing on the neurochemical changes associated with repeated seizures induced by low convulsive dose of pentylenetetrazol (PTZ) (60 mg/kg, sc) in mice. Phenobarbital (10 and 30 mg/kg, ip) significantly diminished the severity of seizures induced by PTZ. Repeated PTZ administration was associated with an increase in [^{3}H]glutamate binding (B_{max} 196.6 ± 10.2 pmol/mg × control B_{max} 137.7 ± 17.0 pmol/mg). Regarding NMDA receptors, repeated PTZ administration was likewise associated with an increase in [^{3}H]MK-801 binding (0.55 ± 0.02 pmol/mg × control 0.32 ± 0.01 pmol/mg). In addition, phenobarbital (10 mg/kg) prevented the increase in [^{3}H]glutamate binding (B_{max} 133.7 ± 11.4 pmol/mg), as well as in [^{3}H]MK-801 binding (phenobarbital 10 and 30 mg/kg, 0.33 ± 0.01 and 0.34 ± 0.01 pmol/mg, respectively). This study reveals an interesting capability of phenobarbital in interfering with the establishment of both the behavioral expression and associated neurochemical changes induced by the repeated administration of low convulsive dose of PTZ, which may be important in the context of preventing epileptogenesis.

Keywords: Antiepileptogenic; Glutamate binding; Pentylenetetrazol; Phenobarbital; MK-801 binding

1. Introduction

Pharmacotherapy of epilepsy utilizes chronic administration of anticonvulsants with the intent to prevent the occurrence of convulsive seizures. Nevertheless, no effective prophylaxis or pharmacotherapeutic cure of epilepsy is currently available [32,47]. Efforts to intervene in the development of epilepsy — epileptogenesis — are in place in the case of patients with brain injury (i.e., through administration of phenytoin and/or carbamazepine) [18,28,44]. It has been argued that preventing epileptogenesis would obviate the need for chronic anticonvulsant drug, and that the use of drugs that inhibit the progression of an epileptic condition would be preferable for patients where epilepsy arises months after initial brain injury [39].

Specific knowledge about epileptogenesis as initiated by trauma, fever, or genetic factors may share new light regarding methods for prophylaxis. Investigations with various animal models have provided experimental evidences for the heterogeneity of events inherent to epileptogenesis; in addition, useful data were provided in clarifying the putative mode, mechanisms and sites of action of chemical agents on epileptogenesis and seizures [40,42,43,45]. In vivo chronic models of epilepsy, in which repeated administration of a subconvulsive electrical or chemical stimulus gradually develops an epileptic state, provides an appropriate approach for quantifying epileptogenesis [31,39].

Repeated administration of small dose of pentylenetetrazol (PTZ) lead to a progressive decrease in seizure threshold, resulting in generalized seizures [19,22]. Once established, such decrease in seizure threshold appears to be permanent for the life of the animal [22,39]. This sort of models, therefore, allows for evaluating neuronal plasticity associated with long-term alterations in neural excitability

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[6], and it has been associated with a increase in susceptibility of the glutamatergic systems [35] resulting in a enhancement of density of glutamate receptors [13,33,34].

Phenobarbital has long been used in the clinic for its antiseizure properties and its therapeutic effectiveness in suppressing the spread of epileptiform seizure activity in the human brain [12,23]. Phenobarbital is relatively nonselective and efficacious in various animal models, inhibits clonic–tonic seizures evoked by electroshock or PTZ, and is able to suppress both electrophysiological and behavioral effects induced by electrical or chemical kindling in rats [2,48]. Phenobarbital also exhibits antiepileptogenic activity on amygdala kindling and PTZ kindling in rats [4,39]. In addition, we have recently reported that phenobarbital prevents the development of PTZ-induced kindling in mice [37].

In the context of the antiepileptogenic and anticonvulsant properties of phenobarbital, the aim of this paper was to investigate the effects of phenobarbital on repeated seizures produced by low convulsive dose of PTZ in mice, focusing on the associated neurochemical changes.

2. Materials and methods

2.1. Animals

Male CF1 mice, 25–30 g, were used throughout the experiments. Mice were kept at 20 ± 2°C, maintained on a 12-h light–dark cycle, with food and water ad libitum. All procedures were carried out according to institutional policies on the handling of experimental animals.

2.2. Drugs and reagents

Phenobarbital was purchased from Sigma. L-[^{3}H]glutamate was purchased from Amersham. [^{3}H]MK-801 was purchased from Du Pont-NEN Products. MK-801 was acquired from RBI, whereas glutamate and glycine were purchased from Merck. All other reagents were of analytical grade.

2.3. Behavioral studies

The procedure is reported in detail elsewhere [37]. Animals (8 weeks old at the start of the experiment) were divided into five groups. Each treatment consisted of two drug administrations repeated once every third day, in a total of six treatments (16 days). Animals were given intraperitoneal injections of phenobarbital 5 mg/kg (Group I; $n = 18$), phenobarbital 10 mg/kg (Group II; $n = 20$), phenobarbital 30 mg/kg (Group III; $n = 24$), or its vehicle (saline solution; group IV; $n = 20$) 30 min prior to convulsive stimuli (60 mg/kg of PTZ, sc). Following each PTZ injection, mice were placed individually in acrylic observation chambers for 30 min and behavioral seizures rated according to the following scale: (0) no convulsive behavior; (1) myoclonic jerks; (2) clonic forelimb convulsions lasting less than 3 s; (3) clonic forelimb convulsions lasting more than 3 s; (4) generalized convulsions with tonic extension episodes. A control group (Group V; $n = 18$) consisted of seizure naive mice that were treated as experimental groups I–IV, except that both injections consisted of saline solution.

Following the 6th treatment (day 16), animals were given a 2-week rest. A further PTZ (60 mg/kg, sc) administration was given alone on day 31, and mice were observed as above. Twenty-four hours after the last testing day mice were decapitated, brains rapidly removed and cerebral cortices dissected and used for the [^{3}H]glutamate and [^{3}H]MK-801 binding assay.

2.4. Neurochemical studies

2.4.1. Membrane preparation

Membranes were prepared as described by Emanuelli et al. [15]. The cerebral cortices were homogenized (20:1 v/w) in 0.32 M sucrose containing 10 mM Tris/HCl buffer (pH 7.4) and 1 mM $MgCl_2$ for [^{3}H]glutamate binding and homogenized (20:1 v/w) in 0.32 M sucrose containing 5 mM Tris/HCl buffer (pH 7.4) for [^{3}H]MK-801 binding assay. All steps were carried out at 4°C. The homogenate was centrifuged twice at $1000 \times g$ for 15 min and the final pellet discarded. Both supernatants were pooled and centrifuged at $27{,}000 \times g$ for 15 min. The supernatant was discarded and the pellet was lysed (20:1 v/w) for 30 min in 10 mM Tris/HCl buffer (pH 7.4). The lysed pellet was washed three times with lysing buffer (20:1 v/w) by centrifuging at $27{,}000 \times g$ for 15 min. Supernatants were discarded and the final pellet was frozen at −70°C for at least 24 h. On the day of binding assay, the membranes were rapidly thawed in a water bath (37°C), homogenized with 3 vol of 10 mM Tris/HCl (pH 7.4), and centrifuged at $27{,}000 \times g$ for 15 min. The resulting pellet was resuspended in the same buffer, preincubated at 37°C for 30 min and centrifuged at $27{,}000 \times g$ for 15 min. The pellet was washed three times in 3 vol of the 10 mM Tris/HCl (pH 7.4), and centrifuged at $27{,}000 \times g$ for 15 min. The final pellet was resuspended in the same buffer in order to yield a protein concentration of 1–2 mg/ml and was used for binding assays. Protein concentration was measured according to the method of Lowry et al. [21].

2.4.2. Binding of [^{3}H]glutamate

For measurement of [^{3}H]glutamate binding, studies were carried out using ligand concentrations in the range from 40 to 3000 nM. [^{3}H]Glutamate was incubated with 100 μg of membrane in 50 mM Tris/HCl buffer (pH 7.4) at 30°C for 15 min in a final volume of 0.5 ml. The reaction was interrupted by centrifugation for 20 min at $27{,}000 \times g$. The supernatant was discarded. The walls of

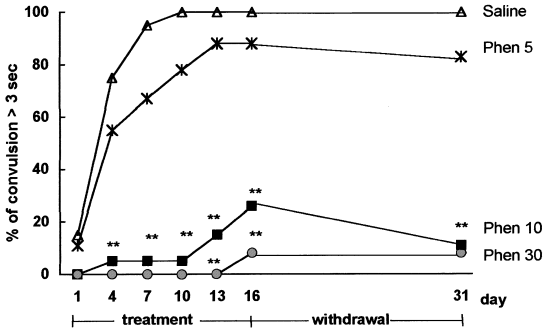

Fig. 1. Percentage of animals presenting severe seizures (clonic seizures lasting more than 3 s) in the saline (△), phenobarbital 5 mg/kg (x), phenobarbital 10 mg/kg (■) and 30 mg/kg phenobarbital (●) groups during repeated administration of PTZ (60 mg/kg, sc) (days 1–16) and after 2 weeks of withdrawal (day 31). $**P<.01$, Fisher test.

the tubes and the surface of the pellets were quickly and carefully rinsed with cold distilled water. The pellets were processed for radioactivity measurement in Beckmam scintillation counter. In order to determine specific binding of [^{3}H]glutamate, each experiment was processed with parallel control tubes containing [^{3}H]glutamate in the presence of 1000 times the amount of unlabeled glutamate. Specific binding was defined as the difference of [^{3}H]glutamate binding between tubes without (total binding) and with (non-specific binding) an excess amount of unlabeled glutamate.

2.4.3. Binding of [^{3}H]MK-801

[^{3}H]MK-801 binding assay was based on the method of Piggott et al. [27]. Briefly, 200 μg of homogenate protein was incubated in 5 mM Tris/HCl buffer (pH 7.4) at 25°C for 1 h, containing 2 nM [^{3}H]MK-801 in the presence of glutamate (50 μM) and glycine (30 μM) in a final volume of 0.5 ml. Non-specific binding was defined as binding, which occurred in the presence of 17,000 times the amount of unlabeled MK-801. After incubation, membranes were filtered under reduced pressure through a Whatman GF/B filters (pre-wetted in 5 mM Tris/HCl buffer), and rinsed rapidly with 3×5 ml ice-cold buffer. The filters were deposited in vials and radioactivity measurement in Beckmam scintillation counter.

2.4.4. Statistical methods

Differences in the percentage of mice presenting seizures with duration ≥ 3 s were evaluated by Fisher's exact test. Differences in seizure scores were evaluated by non-parametric Kruskal–Wallis analysis. Dissociation constant (K_d) and maximal number of specific binding sites (B_{max}) values were estimated by Scatchard analysis. Binding

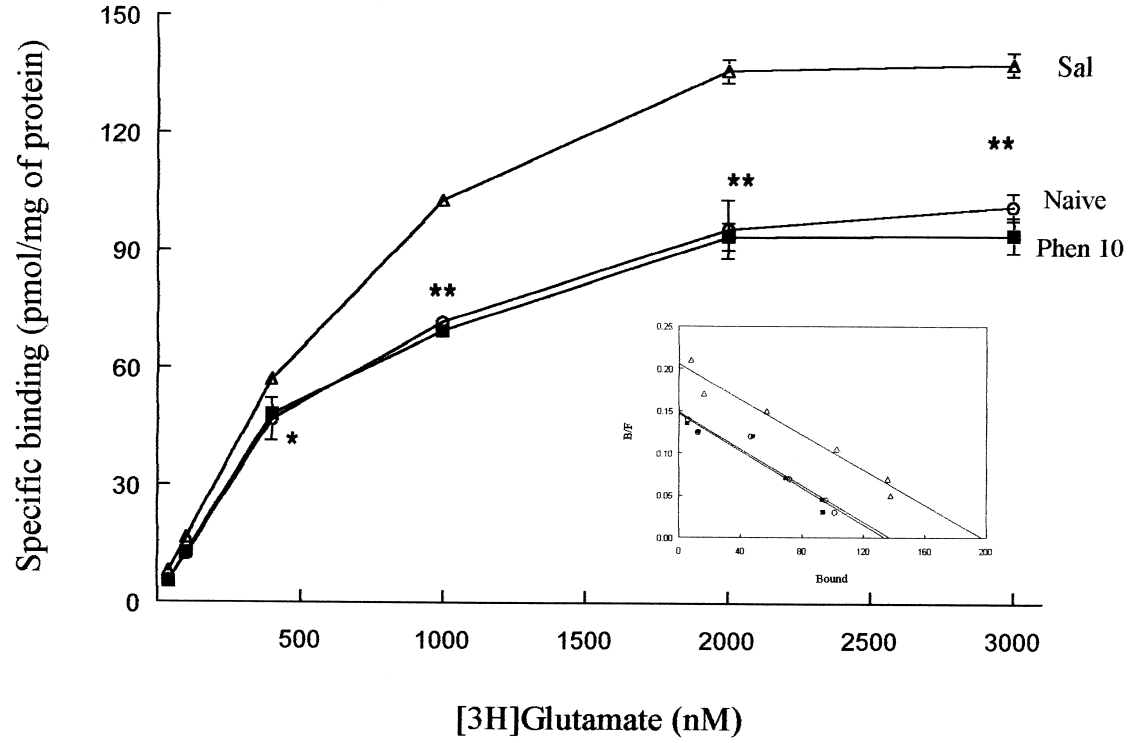

Fig. 2. Effect of saline (△) and phenobarbital 10 mg/kg (■) on L-[^{3}H]glutamate binding (40–3000 nM) of cortical membranes of mice submitted to repeated administration of PTZ (60 mg/kg, sc). Naive (○) mice did not receive PTZ. Values are expressed as specific binding (pmol/mg of protein). Each point represents the mean ± S.E.M. of three separate experiments determined in triplicate (pooled tissues from three to four mice for each membrane preparation). Statistical analysis (ANOVA followed by Duncan test) compared to saline group. $*P<.05$, $**P<.01$.

values were compared by means of two way ANOVA followed by Duncan test.

3. Results

Phenobarbital 10 and 30 mg/kg (but not 5 mg/kg) significantly inhibited the development of severe seizures induced by repeated low convulsive dose of PTZ (Fig. 1) ($P<.01$, Fisher). The mean seizure score on day 16 was: 0 in naive animals; 3.0 ± 0.1 in saline mice; 2.6 ± 0.6 in mice treated with phenobarbital 5 mg/kg; 1.6 ± 0.3 in mice treated with phenobarbital 10 mg/kg ($P<.01$, Kruskal–Wallis); and 0.4 ± 0.1 in mice treated with phenobarbital 30 mg/kg ($P<.01$, Kruskal–Wallis). As shown in Fig. 1, 2 weeks after completion of treatments, the administration of a single PTZ low convulsive dose still resulted in severe seizure in the saline and phenobarbital 5 mg/kg treated animals. Likewise, the antiepileptogenic effect of phenobarbital 10 and 30 mg/kg remained significant ($P<.01$, Fisher). Regarding scores on day 31, the saline-treated group mean score was 3.0 ± 0.1, whereas the mean score of 5, 10, and 30 mg/kg phenobarbital-treated groups were respectively 2.7 ± 0.5 (not significant), 0.8 ± 0.2, and 0.7 ± 0.2 ($P<.01$, Kruskal–Wallis).

Pilot studies indicated that neurochemical data correlates well with behavioral results. As well as observed with seizure severity, [^{3}H]glutamate binding (600 nM) to cortex membranes was found to be significantly ($P<.01$) increased with repeated administration of PTZ (60 mg/kg, sc) in saline (65.9 ± 1.2 pmol/mg) and phenobarbital 5 mg/kg (56.8 ± 3.1 pmol/mg) treated animals as compared with naive (no PTZ) animals (35.2 ± 1.3 pmol/mg; $P<.01$ ANOVA). Accordingly, the increase in [^{3}H]glutamate binding induced by repeated PTZ in saline treated animals (65.9 ± 1.2 pmol/mg) was prevented by treatment with phenobarbital 10 mg/kg (33.0 ± 1.7 pmol/mg; $P<.01$ ANOVA).

Scatchard analysis was performed with phenobarbital 10 mg/kg (Fig. 2). Under the conditions used in this study the binding of [^{3}H]glutamate to cortex membranes from naive mice was characterized by a B_{max} value of 137.7 ± 17.0 pmol/mg and a K_d value of 916.3 ± 96 nM. Repeated administration of PTZ significantly increased [^{3}H]glutamate binding (B_{max} 196.6 ± 10.2 pmol/mg; $P<.01$) without affecting K_d (953.9 ± 26.0 nM). Co-administration of phenobarbital 10 mg/kg prevented the PTZ-induced increase in [^{3}H]glutamate binding (B_{max} 133.7 ± 13.0 pmol/mg) without interfering with its affinity (K_d 914.2 ± 81.0 nM).

Fig. 3 shows [^{3}H]MK-801 binding to mice cortical membranes. Under the conditions used in this study, the binding of [^{3}H]MK-801 (0.32 ± 0.01 pmol/mg) from naive mice was similar to previously published studies [38]. Repeated administration of PTZ significantly increased [^{3}H]MK-801 binding (0.55 ± 0.02 pmol/mg; $P<.01$). Co-administration of phenobarbital 10 and 30 mg/kg prevented the increase in [^{3}H]MK-801 binding (0.33 ± 0.01 and 0.34 ± 0.01 pmol/mg, respectively).

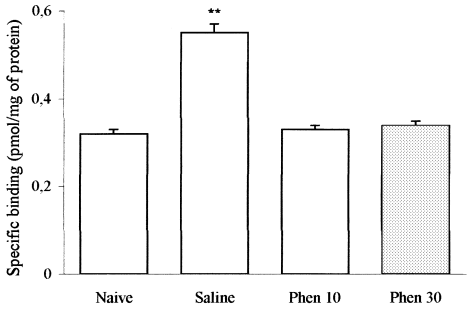

Fig. 3. Effect of saline, phenobarbital 10 mg/kg (Phen 10) and phenobarbital 30 mg/kg (Phen 30) on [^{3}H]MK-801 binding (2 nM) of cortical membranes of mice submitted to repeated administration of PTZ (60 mg/kg, sc). Naive mice did not receive PTZ. Values are expressed as specific binding (pmol/mg of protein). Each point represents the mean ± S.E.M. of three separate experiments determined in triplicate (pooled tissues from three to four mice for each membrane preparation). Statistical analysis (ANOVA followed by Duncan test) compared to naive group. $**P<.01$.

4. Discussion

Our data adds to previous data from electrically and PTZ-induced kindling in rats [4,39] indicating that phenobarbital holds potent anticonvulsant and antiepileptogenic effects. Working with electrically induced kindling in rats, Silver et al. [39] reported that the effective dose of phenobarbital (40 mg/kg) also induced significant ataxia and lethargy. In our study, phenobarbital was effective in doses (10 and 30 mg/kg) that do not induce ataxia or any noticeable motor deficits (animals exhibiting normal exploratory behavior including locomotion, rearing, and grooming). This may be advantageous in distinguishing antiepileptic properties from motor impairments that could eventually disguise the behavioral expression of seizures.

Kindling is an accepted model of experimental epileptogenesis and reflects an altered activity of the excitatory glutamatergic synaptic processes [5,20]. There is extensive evidence indicating diverse roles for ionotropic (NMDA, AMPA/kainate) and metabotropic glutamate receptors in the development and expression of seizures [9,10,17,41]. NMDA receptors were particularly investigated [13,20,30,49], given the effectiveness of its antagonists as anticonvulsants in several animal models [24,25,29]. The ability of NMDA antagonists in blocking kindling development has lead to the suggestion of a potential clinical utility in the prophylaxis of epilepsy [20]. Unfortunately, NMDA antagonists induce severe neurotoxicity, which prevents its clinical use [7,8].

Supporting previous findings [34,37], our data indicate that the density of glutamate binding sites in the cortex was

enhanced in response to repeated seizures induced by low convulsive dose of PTZ. Co-administration of phenobarbital (10 mg/kg) with PTZ prevented the enhancement of [^{3}H]glutamate binding induced by repeated PTZ. In addition, we showed that seizures induced by repeated low dose of PTZ is likewise associated with an increase in [^{3}H]MK-801 binding sites, indicating an increase in density of NMDA receptors [13]. Phenobarbital (10 and 30 mg/kg) prevented the PTZ-induced enhancement of [^{3}H]MK-801 binding. It is noteworthy that drugs able to block the expression of seizures induced by repeated PTZ does not necessarily prevent the associated upregulation of glutamate receptors [14]. Hence, the capability of phenobarbital in preventing both the expression of seizures and the upregulation of glutamate receptors may indicate a genuine antiepileptogenic property.

It has been shown that learning and memory deficits consequent to PTZ-kindling in rats have been differently affected by anticonvulsant agents. Phenobarbital and flunarizine were able to suppress convulsions and deficits, while ethosuximide only suppressed convulsions [3,4]. Glutamatergic transmission, particularly NMDA receptors, seem to be critical to the plastic changes associated with kindling, ultimately leading to decreased seizure threshold and cognitive deficits. Our data support previous evidence that modulation of calcium influx (by drugs affecting glutamate activation or channel blockers as flunarizine) may suppress chronic seizures induced glutamate impairment and its consequences.

The cellular mechanism through which phenobarbital exerts antiepileptogenic action is uncertain. As with other drugs, an enhancement of inhibitory synaptic transmission [36,48] and/or inhibition of excitatory synaptic transmission [24] may be relevant. Impairment of GABAergic inhibition is also thought to play a crucial role in the processes underlying epileptogenesis in the PTZ-kindling model [11,16], and the well-known effects of phenobarbital in facilitating GABAergic transmission is likely to contribute to its antiepileptogenic effect in this model.

It is noteworthy that controlled randomized trials show that prophylactics administration of phenytoin and carbamazepine failed to inhibit the development of post-traumatic epilepsy [18,26,44,50]. These drugs also failed to inhibit development of kindling in animals [1,31,39,46]. If epileptogenesis is dependent upon neural modification common to PTZ-kindling in mice and some forms of human epilepsy, results reported here may have meaningful implications for treating epilepsy.

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