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Acute phenobarbital administration induces hyperalgesia: pharmacological evidence for the involvement of supraspinal GABA-A receptors

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Abstract

The aim of the present study was to determine if phenobarbital affects the nociception threshold. Systemic (1-20 mg/kg) phenobarbital administration dose dependently induced hyperalgesia in the tail-flick, hot-plate and formalin tests in rats and in the abdominal constriction test in mice. Formalin and abdominal constriction tests were the most sensitive procedures for the detection of hyperalgesia in response to phenobarbital compared with the tail-flick and hot-plate tests. The hyperalgesia induced by systemic phenobarbital was blocked by previous administration of 1 mg/kg *ip* picrotoxin or either 1-2 mg/kg *sc* or 10 ng *icv* bicuculline. Intracerebroventricular phenobarbital administration (5 µg) induced hyperalgesia in the tail-flick test. In contrast, intrathecal phenobarbital administration (5 µg) induced antinociception and blocked systemic-induced hyperalgesia in this test. We suggest that phenobarbital may mediate hyperalgesia through GABA-A receptors at supraspinal levels and antinociception through the same kind of receptors at spinal levels.

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- Hyperalgesia
- GABA-A receptors

Introduction

Barbiturates are drugs with the potential to reduce anxiety and promote sleep, to induce general anesthesia, and in special cases to inhibit tonic-clonic seizures (1). In some of these conditions, they have been replaced with other drugs such as benzodiazepines and serotonin re-uptake inhibitors. However, one such drug - phenobarbital - remains the drug of choice for long-term treatment of generalized (tonic-clonic) seizures in view of its effectiveness, low cost and low toxicity (2).

With respect to the mechanism of action, barbiturates are known to enhance the inhibitory effects of the neurotransmitter gamma-aminobutyric acid (GABA) at the GABA-A receptor level (3). To achieve this, barbiturates bind at the GABA-A receptor increasing the opening time of chloride channels, thus permitting chloride ion entry into the cells (4). In addition, activation of the GABA-A receptor has been associated with pain modulation in the central nervous system (CNS), essentially through the descending

inhibitory system (5). However, it is still a matter of discussion in the literature whether barbiturates increase (6,7) or decrease (8,9) the pain threshold.

Since the barbiturate phenobarbital continues to be the drug of choice to treat tonic-clonic seizures, our aim was to investigate if phenobarbital would interfere with the nociceptive threshold. For this purpose, phenobarbital was acutely tested in four algesimetric assays using bicuculline and picrotoxin as pharmacological tools through various routes of administration.

Material and Methods

Animals

Experiments were carried out on male Holtzman rats (180-250 g) and Swiss mice (20-35 g), supplied by the Animal House of the Federal University of Minas Gerais. Animals were housed under controlled temperature (23 ± 2°C), on a 12-h dark/light cycle, with food and water *ad libitum*. The ethical guidelines of the International Association for the Study of Pain for investigations of experimental pain in conscious animals were followed (10).

Measurement of pain threshold

Phenobarbital alone or in combination with the inhibitors picrotoxin or bicuculline was tested in the following methods: tail flick (11), hot plate (12), and formalin (13) in rats, and abdominal constriction (14) in mice. To determine the nociception indices in the tail-flick and hot-plate tests the following formula was used: Tl - Bl/cut-off time - Bl, where Tl and Bl are test latency and baseline latency, respectively. The animals selected for testing were previously submitted to 3 sessions at 10-min intervals with baseline latencies of 3.5-4.5 s (tail flick) and 6-18 s at a temperature of 50°C (hot plate). The cut-off time for the tail-flick and hot-plate tests was 7 and 30 s, respectively. Formalin (1.25%, 50 µl) was injected into one of the hindpaws at time zero. The degree or severity of nociception is described in terms of the animal's behavior and of how it used the injected paw, as follows: degree 0: the paw touches the box, the box wall and floor; degree I: the paw touches the wall and floor lightly and the animal limps; degree II: the paw is not used and does not make contact with any surface; degree III: the animal licks, shakes or bites the paw. The nociception rate (NR) was obtained from the formula: NR = (It + IIt + IIIt/300), where t corresponds to the time (seconds) spent in each degree (I, II or III) during a period of 5 min (or 300 s) for each animal. A full abdominal constriction response to acetic acid (0.6%, v/v) was considered to be present when a wave of contraction followed by extension of the trunk and one hind limb occurred. The number of stretches was recorded during periods of 5 min over 30-min intervals for each animal in the group. The results are reported as mean number of constrictions (± SEM) for each group.

Motor coordination

Phenobarbital was also tested for motor impairment in mice conditioned to a Rotarod apparatus (Ugo Basile). The animals accepted for the test were those who fell from the Rotarod during the first 30 min of observation. The permanence time at 32 rpm was recorded for each animal before (baseline) and 15 min after intraperitoneal (*ip*) drug or vehicle (control) administration. The measures were made in triplicate at 5-min intervals to permit the animals to rest. The mean percentage of reduction (± SEM) in Rotarod permanence time is presented in the Results section.

Intracerebroventricular (*icv*) catheters

The rat was placed in a stereotaxic frame following pentobarbital anesthesia. A hole

was trephined at coordinates overlying the left lateral ventricle, i.e., 1.4 mm posterior to the bregma and 1.5 mm left to the midline, according to the atlas of Paxinos and Watson (15). The guide cannula (an 11-mm long BD-7 stainless steel cannula) was inserted 3-3.3 mm into the lateral ventricle and fixed with a polymerized acrylic helmet adapted to the skull.

Intrathecal (*it*) catheters

Rats undergoing implantation of an *it* catheter were placed in a stereotaxic frame with the head flexed forward. Under pentobarbital anesthesia, a 7-cm long PE-10 tube was inserted into the subarachnoid space through a slit made in the atlanto-occipital membrane and advanced to the level of the lumbar spinal cord. The external part of the catheter was tunnelled into the skull to exit on the parietal bone. The catheter was fixed with a small piece of acrylic placed between the atlanto-occipital membrane and the skull (16).

Drug treatment

Phenobarbital was diluted in vehicle consisting of propylene glycol:saline (50:50, v/v) and administered *ip* 20 min before the beginning of the test or either *it* or *icv* 20 min after the beginning of the test. Picrotoxin and bicuculline were dissolved in physiological saline and administered systemically by the *ip* or *sc* route 30 min before the test. Only bicuculline was administered centrally by the *icv* or *it* route 20 min after the beginning of the test. When the drug was administered systemically (*ip*) the volume used was 0.1 ml/100 g for rats and 0.1 ml/10 g for mice. For the *icv* and *it* injections the volume used was 10 µl 7-10 days after cannula implantation. *Icv* and *it* injections of drugs were carefully performed over a period of 90 s to avoid intracranial hypertension or drug extravasation.

Drugs and vehicles

The following drugs were purchased from the stated sources: picrotoxin and bicuculline (Sigma Chemical Co., St. Louis, MO, USA), phenobarbital (Rhodia, Santo Amaro, SP, Brazil), propylene glycol (Reagen, Rio de Janeiro, RJ, Brazil), acetic acid (Merck, Rio de Janeiro, RJ, Brazil) and formaldehyde (38%; Labsynth, Diadema, SP, Brazil). Formaldehyde was mixed with saline to obtain a final 1.25% formalin concentration.

Statistical analysis

The results are reported as mean ± SEM. Data for the treatment groups were compared by Kruskal-Wallis analysis of variance (ANOVA) on ranks followed by the Dunnett test for multiple comparisons of nonparametric data. The level of significance was set at $P<0.05$ and the analyses were performed using the Sigma Stat software (version 1.199).

Results

Experiment I

Different doses of phenobarbital (2.5, 5, 10 and 20 mg/kg) or vehicle were injected *ip* into rats to evaluate the effects of the drug on the latency for the tail-flick reflex and the hot-plate test, as well as the nociception rate in the formalin test. In addition, the abdominal stretches in response to acetic acid were counted in mice pretreated with different doses of phenobarbital.

Systemic administration of different doses of phenobarbital (5-20 mg/kg) induced a dose-dependent reduction of the latency response in rats compared with control animals (vehicle) in the tail-flick and the hot-plate test, indicating development of hyperalgesia (Figure 1A and B; $P<0.05$, Kruskal-Wallis test). In addition, phenobarbital (1.25-5 mg/kg) dose dependently increased the

pain score of the formalin test in rats (Figure 1C) as well as the stretch number in mice, also indicating the development of hyperalgesia (Figure 1D). A time between 40 and 60 min was necessary to observe the maximal hyperalgesic effect in the tail-flick and hot-plate tests. The maximal hyperalgesic effect occurred at 10 and 25 min after phenobarbital administration in the constriction test and formalin test, respectively. Specifically, phenobarbital increased the nociception rate both in phases 1 and 2 of the formalin-induced response (Figure 1C).

However, the minimal dose of phenobarbital needed to induce hyperalgesia varied according to the test used: 1.25 mg/kg and 2.5 mg/kg were enough to induce a significantly increased effect in the formalin and constriction tests, whereas a 2- to 8-fold increase in the dose was necessary to induce hyperalgesia in the hot-plate and the tail-flick test, respectively. In addition, reduction in the time of permanence in the Rotarod apparatus was observed in mice with increasing doses of phenobarbital (1-35 mg/kg), indicating a dose-dependent loss of motor coordination following hyperalgesia (Table 1).

Experiment II

A previous (15 min) dose of 1 mg/kg picrotoxin blocked the hyperalgesia induced by phenobarbital in all algesimetric assays performed ($P<0.05$, Kruskal-Wallis test). This dose of picrotoxin induced a significant

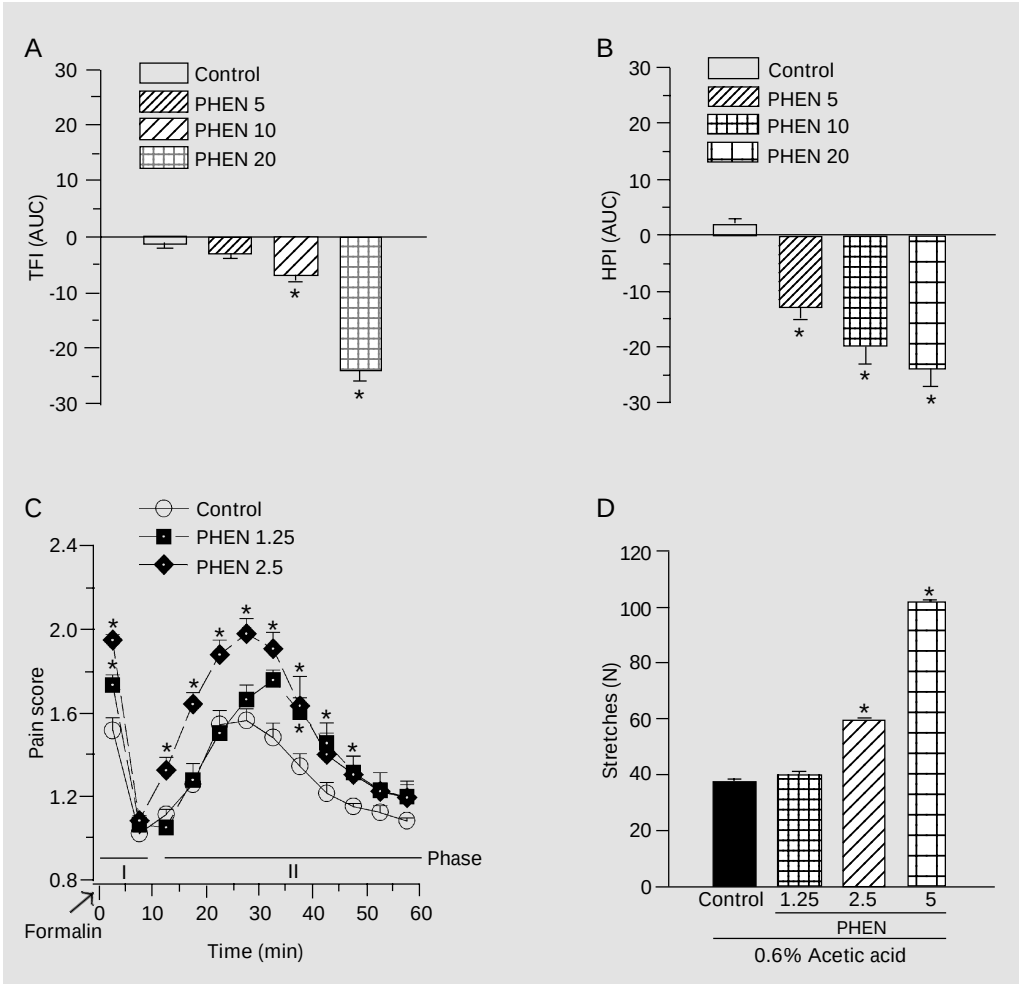

Figure 1 - Dose-dependent hyperalgesia induced by acute phenobarbital (PHEN) treatment of rats as measured by the tail-flick (A), hot-plate (B), and formalin tests in rats (C) and the abdominal constriction test induced by acetic acid in mice (D). Phenobarbital (1.25-20 mg/kg) was administered *ip* 20 min before the test (N = 8). Control (vehicle) and phenobarbital-treated animals were injected with the following volumes: 0.1 ml/100 g for rats and 0.1 ml/10 g for mice. Results are reported as mean area under the curve (AUC) for tail-flick index (TFI) and hot-plate index (HPI) ± SEM, in A and B, respectively, during 60 min of observation. The response to the formalin test (1.25%) is illustrated (phases I and II) in the corresponding graph (C), and the number of stretches/30 min following *ip* acetic acid administration is shown in D. Results are reported as mean ± SEM. *P<0.05 compared to control (Kruskal-Wallis test followed by Dunnett's multiple comparisons test).

antinociceptive effect when administered alone, which could be observed in the four tests studied (Figure 2A-D). Lower doses of picrotoxin (0.12 to 0.5 mg/kg) also blocked phenobarbital-induced hyperalgesia, but did not induce an antinociceptive effect *per se* (data not shown). Surprisingly, however, phenobarbital increased the antinociceptive effect of picrotoxin in two algesimetric tests performed in combination, i.e., the tail-flick (Figure 2A) and hot-plate (Figure 2B) tests.

An antinociceptive dose of systemic bicuculline (1-2 mg/kg, *ip*) blocked the hyperalgesia induced by phenobarbital both in the tail-flick and constriction tests (Figure 3A and B, respectively). In addition, phenobarbital in combination with bicuculline also potentiated the antinociceptive effect induced

Table 1 - Reduction of permanence time (%) in the Rotarod apparatus for mice treated with various doses of phenobarbital.

Various doses of phenobarbital were administered *ip* to mice submitted to rotation (32 rpm) in a Rotarod apparatus (Ugo Basile). The animals were previously adapted to the experimental conditions and the permanence time in the apparatus was recorded during 5-min intervals over a period of 30 min. The results are reported as mean ± SEM and the number of animals is given in parentheses.

Dose (mg/kg)	% Reduction in permanence time
Control	-18.0 ± 2.58 (6)
1	16.7 ± 1.65 (6)
5	37.4 ± 1.52 (5)
10	48.9 ± 6.26 (5)
20	61.5 ± 2.16 (5)
30	74.23 ± 1.13 (5)
35	88.89 ± 1.55 (5)

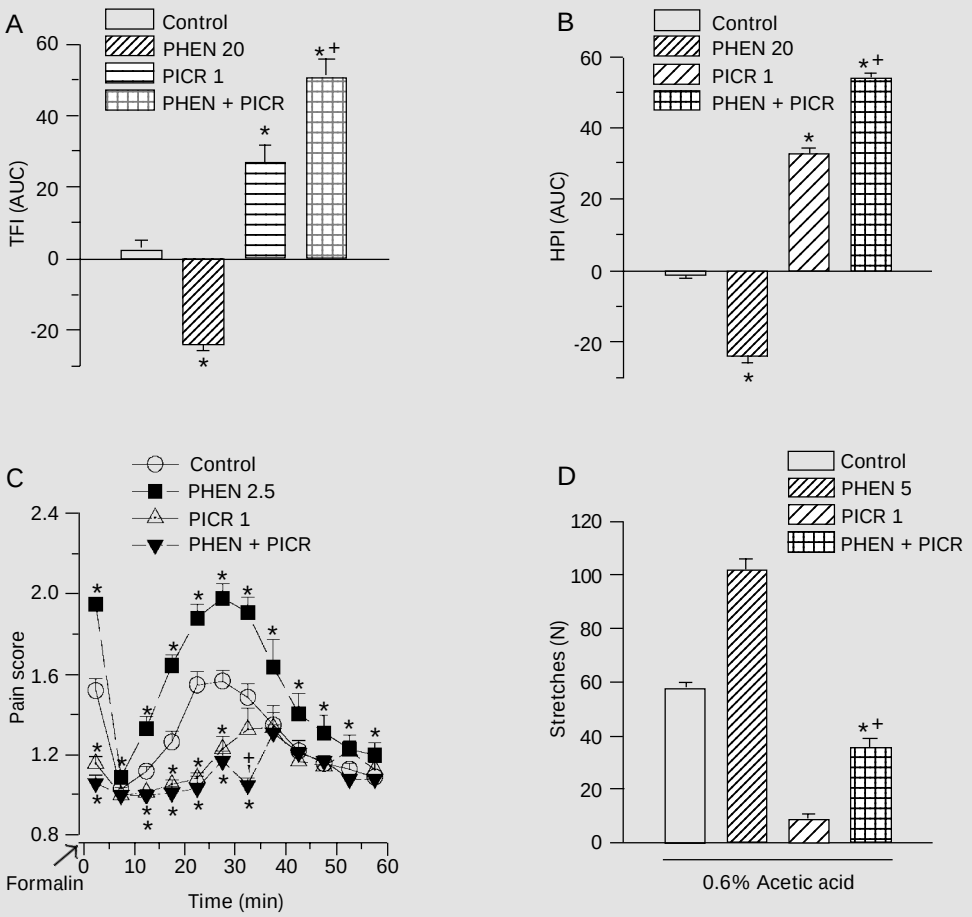

Figure 2 - Blockade by picrotoxin of phenobarbital-induced hyperalgesia in rats (A, B, C) and mice (D). Picrotoxin (PICR; 1 mg/kg) was administered *ip* 10 min before *ip* phenobarbital (PHEN; 2.5-20 mg/kg) administration. Hyperalgesia (decrease of nociception threshold, N = 8 animals/group) and its consequent blockade (antinociception) were detected in the tail-flick (A) and hot-plate tests (B), reported as area under the curve (AUC) for tail-flick index (TFI) and hot-plate index (HPI) and in the formalin (C) and abdominal constriction (D) tests, reported as nociception rate and number of stretches, respectively. *P<0.05 compared to control (animals injected with vehicle), and $^{+}$P<0.05 compared to picrotoxin-induced antinociception (Kruskal-Wallis test followed by Dunnett's multiple comparisons test).

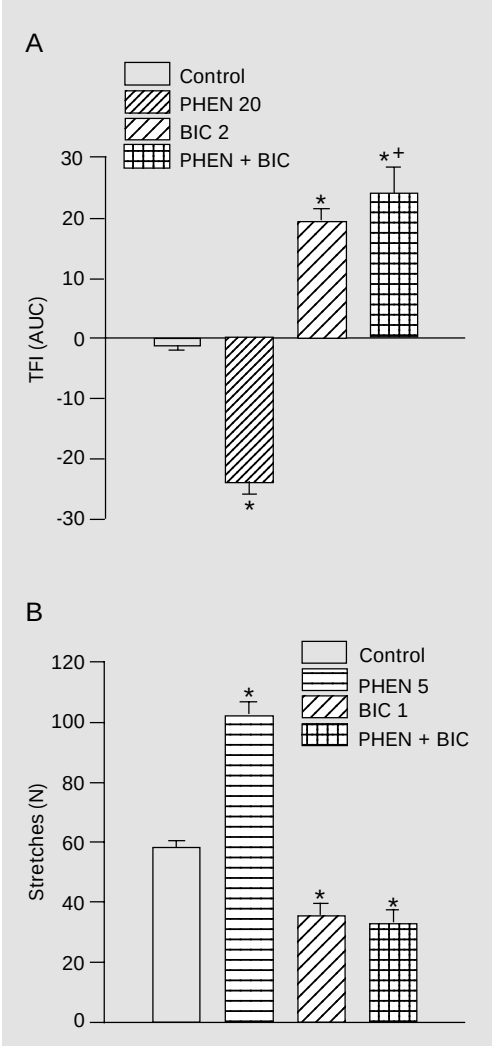

Figure 3 - Reversal of phenobarbital (PHEN)-induced hyperalgesia by systemic administration of bicuculline (BIC) in the tail-flick test in rats and the abdominal constriction test induced by acetic acid in mice. Bicuculline (1 or 2 mg/kg) was administered *sc* 10 min before *ip* administration of phenobarbital. Control animals were injected with the respective vehicles at the times indicated. Results are reported as mean ± SEM (N = 8). *P<0.05 compared to control and $^{+}$P<0.05 compared to bicuculline-induced antinociception (Kruskal-Wallis test followed by Dunnett's multiple comparisons test).

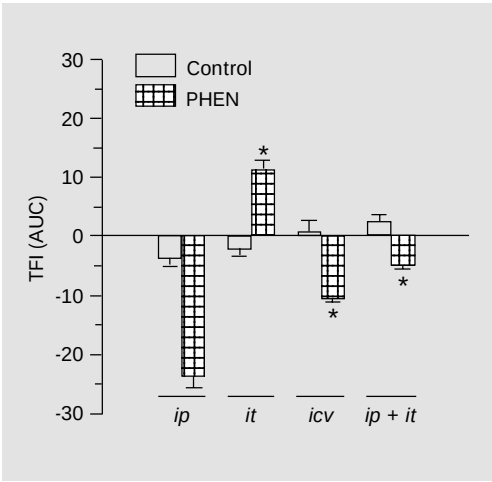

Figure 4 - Opposite (hyperalgesic or antinociceptive) effects of phenobarbital (PHEN) administration by the *icv* and *it* routes in the tail-flick test. Inhibition by *it* administration of phenobarbital of the hyperalgesia induced by systemic phenobarbital administration is also shown (*ip* + *it*). A phenobarbital dose of 5 µg/site was used for CNS (*icv* and *it*) administration. Systemic phenobarbital (20 mg/kg, *ip*, N = 5) was administered alone 10 min before *it* administration. *P<0.05 compared to systemic administration (Kruskal-Wallis test).

by the latter in the tail-flick test (Figure 3A).

Experiment III

Phenobarbital was used at a dose of 5 µg by the *icv* or *it* route to study the participation of the CNS in its hyperalgesic effect, using the tail-flick method. Intracerebroventricular administration of phenobarbital (5 µg) to rats induced development of hyperalgesia as detected by the tail-flick test (Figure 4). In contrast, the same dose of phenobarbital administered *it* induced an antinociceptive effect, as shown in the same figure. In addition, *it* administration of phenobarbital reduced the hyperalgesia induced by its systemic administration (Figure 4).

Intracerebroventricular administration of bicuculline (10 ng) blocked both the hyperalgesic effect induced by systemic phenobarbital treatment (Figure 5) and the antinociceptive effect induced by *it* phenobarbital administration (data not shown).

Discussion

We have previously shown that various anxiolytic drugs interfering with the GABA-A receptor, including barbiturates, could induce a state of hyperalgesia (7), although the literature about this subject may be considered controversial (8,9). Extending data from previous work (7), in the present study we showed that acute phenobarbital administration induced a dose-dependent hyperalgesia. This hyperalgesia was detected in four algesimetric assays, i.e., the tail-flick, hot-plate and formalin tests in rats, and in the abdominal constriction test in mice, thus indicating a clear-cut effect. Such effect was still more evident if one considers that at all phenobarbital doses tested a reduction of motor coordination occurred in the animals, a fact that may have theoretically impaired the observations leading to erroneous conclusions, such as a pseudo-"antinociceptive" effect, especially in mice. In fact, it should

be pointed out that if higher doses of phenobarbital (>5 mg/kg) were used in the formalin or constriction test, hyperalgesia would be probably reduced by the sedation presented by the animals.

Some barbiturates are still used today therapeutically as general anesthetics (thiopental) and anticonvulsants (phenobarbital). These drugs essentially act at the CNS level, facilitating GABAergic neurotransmission by binding at specific sites in the GABA-A receptor (4). Besides controlling seizures, GABA-A receptor activation may also be involved in pain modulation (17-19). The principal nuclei of pain neuromodulation in the CNS are the periaqueductal gray matter, nucleus raphe magnus and the spinal dorsal horn which constitute the descending inhibitory system. It has been shown that stimulation of GABAergic (inhibitory) neurones associated with the periaqueductal gray matter and nucleus raphe magnus may increase the painful afferent inputs coming from the periphery (19,20), whereas activation of GABAergic neurones at the spinal dorsal horn level may have the opposite effect, i.e., a decrease of the painful peripheral inputs (21).

The potency of phenobarbital in inducing hyperalgesia varied between tests. It has been described long ago that the tail-flick response is thought to be a spinal reflex, while the hot-plate involves at least the brainstem level since coordination of the head and limbs is necessary for the response to be observed (22). The same authors also stated that the more complex behavioral pattern of the formalin test might involve other brain regions in addition to those involved in a rapid flick of the tail. Electrophysiological studies have shown that analgesia produced by stimulation of the periaqueductal gray matter requires a significantly lower current intensity in the formalin test than in the tail-flick test (23). From a pharmacological point of view, these data may suggest that a hyperalgesic dose of phenobarbital is smaller in an algesimetric assay of higher complexity. In addition, our results also support the notion that the hyperalgesic effect induced by phenobarbital is not restricted to one species, since the response was consistently detected in rats and mice.

To test if GABA-A receptors could be involved in the hyperalgesic response induced by phenobarbital, we initially chose picrotoxin - a convulsive substance which blocks the chloride channel associated specifically with the GABA-A receptor (4). Indeed, subconvulsant doses of picrotoxin inhibited the phenobarbital-induced hyperalgesic response in all tests used. This antinociceptive effect of picrotoxin has been previously observed by Tatsuo et al. (7) and may derive from an action through the GABA-A receptors present in the descending inhibitory system.

The most striking result, however, was the potentiation of the picrotoxin-induced antinociceptive response when the drug was combined with phenobarbital treatment observed in 2 out of 4 tests used, i.e., the tail-flick and hot-plate test. In fact, when 1 mg/kg picrotoxin was used in the formalin test this effect was not so clear-cut (Figure 3C), but when a lower dose was used (0.12 mg/kg) this effect could be clearly demonstrated

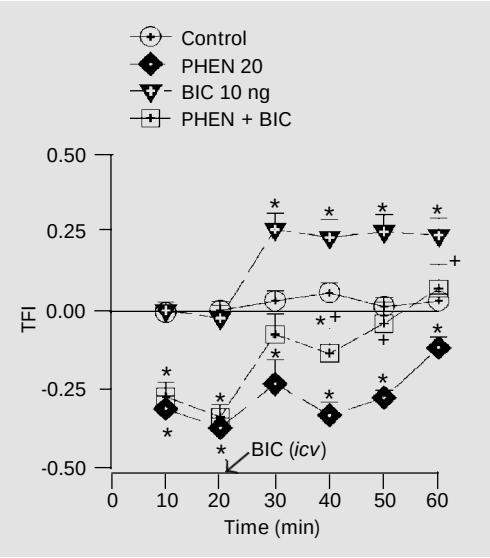

Figure 5 - Antinociceptive effect of bicuculline (BIC) following its acute *icv* administration to rats. The inhibition by bicuculline (10 ng, N = 5) of the hyperalgesic effect induced by systemic (*ip*) phenobarbital (PHEN) administration (20 mg/kg, N = 5) is also shown. Control animals were injected with the same volume of vehicle as used for the bicuculline and phenobarbital-treated animals. *P<0.05 compared to control and $^{+}$P<0.05 compared to bicuculline-induced antinociception (Kruskal-Wallis test followed by Dunnett's multiple comparisons test).

(Yokoro CM and Tatsuo MAKF, unpublished observations). Some studies have shown that the site of barbiturate binding is close to the site of the neurotransmitter binding in the GABA-A receptor (4,5). On the other hand, the picrotoxin site is inside the channel where it seems to inhibit the entry of the chloride ions into the cell (24). Since phenobarbital potentiated the antinociceptive action of picrotoxin, it is our hypothesis that the binding of phenobarbital to its active site would allosterically change the GABA-A receptor, increasing the chance of picrotoxin binding at its site inside the channel.

Finally, the hyperalgesic effect induced by phenobarbital seems to derive from an action on upper rather than spinal levels, since hyperalgesia was observed when phenobarbital was injected by the *icv* but not by the *it* route. The fact that spinally administered phenobarbital induced an opposite (antinociceptive) effect as demonstrated in the present study, even blocking the hyperalgesia induced by (systemic) phenobarbital itself, supports this concept. Differences in subunit composition of the GABA-A receptor in supraspinal and spinal neurones (25) could account for the differences observed in the action of phenobarbital at the molecular level. This hypothesis was also supported by the demonstration that the specific GABA-A receptor antagonist bicuculline induced antinociception by the *icv* route in the tail-flick test (present study). Further studies from our laboratory have shown that *it* bicuculline administration potentiated the hyperalgesia induced by systemic administration of phenobarbital itself (Yokoro CM and Tatsuo MAKF, unpublished observations).

In conclusion, the anticonvulsant drug phenobarbital when acutely administered induced hyperalgesia within a dose range of 1.25 to 20 mg/kg in four experimental pain assays. This effect was completely blocked by subconvulsant doses of picrotoxin and bicuculline, the antagonists acting on the chloride channels associated with the GABA-A receptor and the specific receptor for the inhibitory neurotransmitter GABA, respectively, clearly implicating GABA-A receptors in this hyperalgesic effect of phenobarbital. The site for this hyperalgesic response seems to be linked with upper rather than spinal levels in the CNS, since 1) phenobarbital induces hyperalgesia when administered by the *icv* route, reproducing the hyperalgesic effect induced by its systemic administration; 2) *it* administration of the drug induces an opposite effect, i.e., an antinociceptive effect; 3) the competitive GABA-A antagonist bicuculline alone induced the opposite effect of the agonist phenobarbital, i.e., antinociception, when administered systemically (*ip*) or *icv*, and 4) bicuculline in combination with phenobarbital blocked the hyperalgesic effect induced by the latter. However, phenobarbital administered by the *it* route induced antinociception. Since GABA-A receptors are associated with modulation of the descending inhibitory system at upper levels of the CNS, we suggest that the phenobarbital-induced hyperalgesic response may derive from an inhibitory effect on this system, favoring the painful inputs coming from the periphery. We also suggest that phenobarbital may induce an antinociceptive effect through an effect at the spinal level also involving GABA-A receptors.

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