**Pharmacological Reports**
2012, 64, 305–314
ISSN 1734-1140



# Influence of calcium channel blockers on anticonvulsant and antinociceptive activities of valproic acid in pentylenetetrazole-kindled mice

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**Abstract:**
**Backgroud:** Comorbidities of epilepsy comprise some pain disorders, including acute nociceptive pain, therefore, antiepileptic drugs can prove efficacy in the management of this kind of pain albeit with several adverse reactions. The current study aimed to evaluate the modulatory effects of calcium channel blockers on the anticonvulsant and antinociceptive effects of valproic acid (VPA) in pentylenetetrazole (PTZ)-kindled mice.
**Methods:** Kindled mice were treated with 20 mg/kg (*ip*) of diltiazem, nifedipine, or verapamil, then VPA (200 mg/kg, *ip*) at 30 min intervals before PTZ administration (35 mg/kg, *ip*).
**Results:** Our data demonstrated that the three calcium channel blockers afforded a protection against sub-convulsive doses of PTZ. Their protective effects were comparable to that exerted by the standard antiepileptic drug, VPA. The anticonvulsant activity of VPA was further enhanced by its combination with diltiazem. Also, PTZ-kindling reduced pain-threshold as evaluated by hot plate analgesimeter and acetic acid-induced writhing test. Although the repeated administration of VPA significantly increased pain-threshold in kindled mice, it was not able to normalize it. Similar results were obtained with diltiazem and nifedipine. Interestingly, combination of diltiazem or nifedipine with VPA elicited the most profound antinociceptive effect in kindled mice.
**Conclusions:** These results demonstrate for the first time the beneficial role of some calcium channel blockers in combination with VPA in the management of acute nociceptive pain. Therapeutically, this enhancing profile for calcium channel blockers fosters a safer and more effective drug-combination regimen than valproic acid alone.

**Key words:**
diltiazem, epilepsy, kindling, nifedipine, nociception, pentylenetetrazole, valproic acid, verapamil

**Abbreviations:** PTZ – pentylenetetrazole, VPA – valproic acid

## Introduction

Epilepsy is one of the most common neurologic disorders, with prevalence estimates for active epilepsy ranging from 0.2–4.1% [6]. A number of conditions have been reported to be comorbid with epilepsy, including certain pain disorders [43].

Pain is an unpleasant sensory and emotional experience that is associated with actual or potential tissue damage. Pain can be classified as acute or chronic based on the length of time the pain is experienced. Acute pain, resulting from tissue damage, is a normal physiological response and serves a protective function. Nociceptive pain is typically acute in nature and diminishes upon healing, while chronic pain is an ab-

normal sensation usually occurring after direct injury or damage to a nerve. For individuals experiencing chronic pain, their pain usually includes inflammatory and neuropathic pain [45, 58]. Similar pathophysiological and biochemical reactions were detected in neuropathic pain syndromes and epilepsy [61]. Therefore, antiepileptic drugs can prove their efficacy in the management of this condition.

Most of antiepileptic drugs can reduce neuronal hyperexcitability by inhibiting ion channels, although they may act simultaneously on different parts of the nociceptive pathway [61]. Valproic acid (VPA) is widely used in the management of epilepsy, bipolar disorders, social phobias, and neuropathy [29]. VPA causes an increased concentration of natural inhibitor, GABA, in central nervous system (CNS) synapses [12] and hence is used for the treatment of chronic intractable pain [13]. Unluckily, VPA is known to induce several adverse reactions including hemorrhagic pancreatitis, bone marrow suppression and hepatotoxicity [7, 18]. Moreover, VPA toxicity that can seriously mount to death has also been reported [48]. Therefore, diverse concepts have been adopted to subside such VPA's toxicity, which merely focused on lessening it either by enhancing the mitochondrial fatty-acyl transport for its β-oxidation products or combating oxidative stress [34, 47]. On the contrary, little, if any, attempts have targeted enhancing the pharmacologic efficacy of VPA so as to reduce its effective dose, hence also toxicity.

Calcium ion is a regulator of metabolic pathways and serves important functions as a second messenger. Calcium ion influx occurs by voltage dependent and/or receptor-operated calcium channels [32]. A calcium ion flux into the intracellular space represents the first stage of epileptic neuronal events [10]. The initiation of epileptogenic activity in the neuron is thought to involve the normal phenomenon known as "intrinsic burst firing" that is activated by an inward calcium ion current [16]. It was demonstrated that calcium ion flux into the pre-synaptic terminal is an important factor for neurotransmission [46]. The blockade of different types of calcium ion channels was proposed as a possible mechanism of action of VPA [9, 51]. Furthermore, epileptic depolarizations of neurons were found to be depressed by calcium ion channel blockers [23, 46].

Accordingly, we aimed in the current study to evaluate the modulatory effects of three calcium channel blockers; diltiazem, nifedipine, or verapamil, on the anticonvulsant and antinociceptive activities of VPA in pentylenetetrazole (PTZ)-kindled mice.

## Materials and Methods

### Experimental animals

All animal procedures and the experimental protocols were approved by the research and ethics committee of Suez Canal University. Two hundred and twenty four male Swiss albino mice (weighing 22–26 g) were obtained from the Egyptian Organization for Biological Products and Vaccines (Vacsera, Egypt) and housed under controlled conditioning (25 ± 1°C temperature, 55–65% relative humidity, 12 h dark/light cycles). After 7 days of acclimatization to laboratory conditions, the animals were randomly assigned to experimental groups, 14 mice each. Food and water were allowed *ad libitum* during the study period. All tests were performed between 9:00 a.m. and 3:00 p.m. to minimize circadian influences on seizure susceptibility.

### Chemicals and drugs

The following drugs were used in the current study: nifedipine (Epico Pharmaceutical Industrial Co.), diltiazem and verapamil (Sigma Pharmaceutical Industrial Co.). VPA (sodium salt), PTZ and all other chemicals were obtained from Sigma (St. Louis, MO, USA).

### Pharmacological treatments

Animals were divided into 16 main groups, 14 mice each. All calcium channel blockers (diltiazem, nifedipine, and verapamil) were suspended in a 1% aqueous solution of Tween 80 and administered intraperitoneally (*ip*) at a dose of 20 mg/kg of body weight [40]. Fresh drug solutions were prepared on each day of experimentation and administered with or without VPA (200 mg/kg) 30 min before PTZ administration, a time proven to allow peak plasma VPA level to be reached [24]. The experimental design is summarized in Table 1.

**Tab. 1.** Summary of experimental design

| No. | Control groups | No. | Kindled groups |
|---|---|---|---|
| 1 | Normal | 2 | PTZ |
| 3 | VPA | 4 | PTZ + VPA |
| 5 | Diltiazem | 6 | PTZ + Diltiazem |
| 7 | Nifedipine | 8 | PTZ + Nifedipine |
| 9 | Verapamil | 10 | PTZ + Verapamil |
| 11 | VPA + Diltiazem | 12 | PTZ + VPA + Diltiazem |
| 13 | VPA + Nifedipine | 14 | PTZ + VPA + Nifedipine |
| 15 | VPA + Verapamil | 16 | PTZ + VPA + Verapamil |

First column shows the control groups corresponding for each kindled group. Second column shows kindled groups which received sub-convulsive doses of pentylenetetrazole (PTZ, 35 mg/kg, *ip*) every other day, for a total of 11 injections, either alone or in different combinations with valproic acid (VPA, 200 mg/kg, *ip*), diltiazem (20 mg/kg, *ip*), nifedipine (20 mg/kg, *ip*), or verapamil (20 mg/kg, *ip*). Treatments with VPA and/or calcium channel blockers were commenced 30 min before each PTZ injection. At the end of experiment, i.e., after the last dose of PTZ, all groups were divided into 2 subgroups, a and b, 7 mice each. These subgroups were used for hot plate test and acetic acid-induced writhing test, respectively

### Kindling induction and staging

For kindling induction, PTZ was freshly dissolved in normal saline and a sub-convulsive dose (35 mg/kg, *ip*) was administered every other day, for a total of 11 injections [27]. After each injection of sub-convulsive dose of PTZ, mice in different groups were observed for 30 min and PTZ-induced seizures were evaluated and classified according to the scoring system of Fischer and Kittner [20]: 0 – no evidence of convulsive activity; 1 – ear and facial twitching, head nodding; 2 – myoclonic jerks; 3 – forelimb clonuses, full rearing; 4 – generalized clonic convulsions rearing, jumping, falling down, loss of righting reflex; 5 – clonic-tonic convulsions, tonic hindlimb extensions.

The mean seizure stages were calculated for all groups after each PTZ injection.

### Hot plate method

Hot plate is one of the most commonly used methods for the evaluation of antinociceptive drug effect. In the current study, the effect of tested drugs on nociceptive thresholds was assessed in all subgroups "a" using the mouse hot plate analgesimeter by evaluating the reaction time of each mouse when placed on a hot plate (Panlab LE 7406, Spain) maintained at 55°C. Before the administration of test compounds, the baseline hot plate latency, defined as licking or jumping, of each mouse was determined. After treatment, a maximal possible latency of 60 s was allowed, after which mice not responding were removed from the hot plate surface to avoid tissue damage [33]. For the determination of maximum possible effects, hot plate test was performed 6 times (at 30, 60, 90, 120, 150 and 180 min) post-PTZ injection in the same animals. The nociceptive thresholds at 120 min were the minimal in the current study; therefore, the 120 min results were expressed.

### Acetic acid-induced writhing

Two hours after the last dose of PTZ, an acetic acid solution (0.8%; 0.1 ml/10 g body weight) was administered *ip* to all subgroups "b". After a further 10 min, the number of constrictions was recorded for another 10 min [17].

### Statistical analysis

All data were expressed as the mean ± SEM. Statistical significance was tested by one way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* analysis. The confidence limit of $p < 0.05$ was considered statistically significant.

## Results

### Kindling stage

Repeated treatment with PTZ at a sub-convulsive dose, every other day for a total of 11 injections, induced chemical kindling (Fig. 1). Our data demonstrated that single treatment with the antiepileptic drug VPA or any of the calcium channel blockers; diltiazem, nifedipine, or verapamil, 30 min before PTZ injections significantly decreased the kindling stage compared to PTZ-treated mice. Similar results were obtained when VPA was administered concurrently with diltiazem, nifedipine or verapamil, where a significant ($p < 0.05$) reduction in kindling stage was ob-

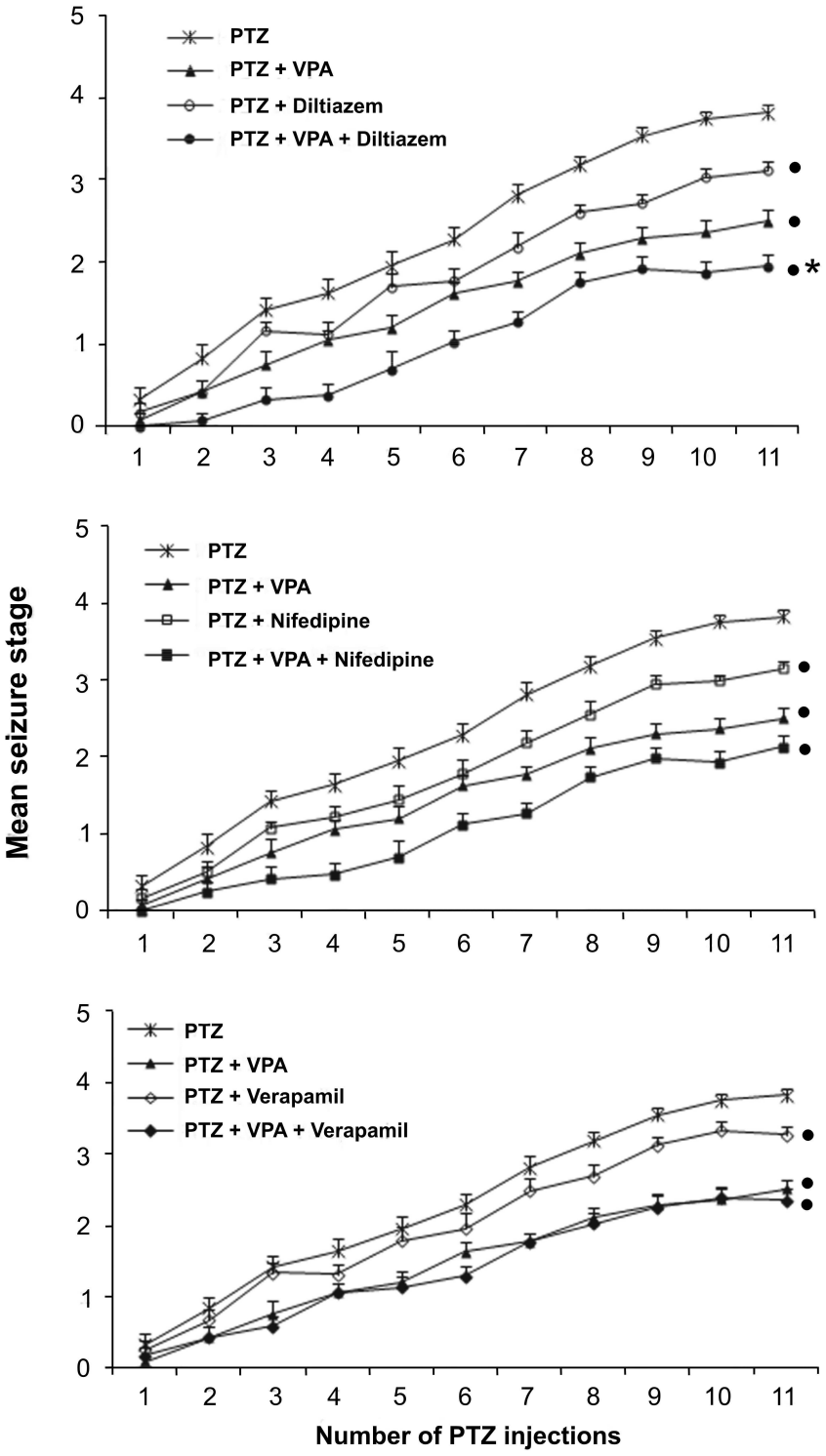


**Fig. 1.** Effects of daily *ip* administration of 20 mg/kg of diltiazem (upper panel), nifedipine (middle panel), or verapamil (lower panel) either alone or in combination with VPA (VPA, 200 mg/kg), on the development of PTZ-induced kindling (35 mg/kg, *ip*, every other day, for a total of 11 injections) in mice. Mice were scored using Fischer and Kittner seizure stage rating scale. Data are presented as the mean seizure stage ± SEM. All data were analyzed using ANOVA followed by Bonferroni *post-hoc* test; • $p \leq 0.05$ with respect to PTZ, * $p \leq 0.05$ with respect to PTZ + VPA. Each group consisted of 14 mice

served in comparison with untreated kindled group. Unlike nifedipine and verapamil, co-administration of diltiazem with VPA showed significant ($p < 0.05$) reduction in the kindling stage compared to valproic acid-treated kindled group (Fig. 1).

### Hot plate test

Effects of administration of PTZ, valproic acid, or calcium channel blockers on the cut time of the hot plate test were evaluated in the current study (Fig. 2).

**Fig. 2.** Effects of daily *ip* administration of 20 mg/kg of diltiazem (upper panel), nifedipine (middle panel), or verapamil (lower panel) either alone or in combination with VPA (VPA, 200 mg/kg), on the cut time (s) of the hot plate test (55°C) in control as well as PTZ-kindled (35 mg/kg, *ip*, every other day, for a total of 11 injections) mice. Values were obtained at 120 min after last PTZ injection and expressed as the mean ± SEM. All data were analyzed using ANOVA followed by Bonferroni *post-hoc* test; • $p \leq 0.05$ with respect to control, * $p \leq 0.05$ with respect to PTZ, # $p \leq 0.05$ with respect to VPA, ° $p \leq 0.05$ with respect to corresponding control. Each group consisted of 7 mice

Our results indicated that sub-convulsive doses of PTZ decreased the cut time of the hot plate test significantly ($p < 0.05$) compared to control group. Treatment of control mice with anti-epileptic drug, valproic acid, or calcium channel blockers, diltiazem or nifedipine significantly ($p < 0.05$) increased the cut time when compared to control group. On the other hand, treatment with verapamil, showed no significant difference from control group. Moreover, administration of valproic acid, diltiazem or nifedipine to kindled mice significantly ($p < 0.05$) increased the cut time compared to PTZ-treated group to restore it to normal level. In contrast, administration of verapamil showed no significant difference from kindled group. Furthermore, the results indicated that co-administration of VPA with any of the three calcium channel blockers increased the cut time dramatically compared to control group and restored the cut time to normal level in kindled groups. It is also observed that combining diltiazem or nifedipine with VPA significantly ($p < 0.05$) increased the cut time compared to VPA treated group, the effect that was not shown with verapamil.

### Acetic acid writhing test

Effects of administration of PTZ, valproic acid, or calcium channel blockers on the number of writhings have been also investigated. Current results indicated that sub-convulsive doses of PTZ increased the number of writhings significantly ($p < 0.05$) in com-

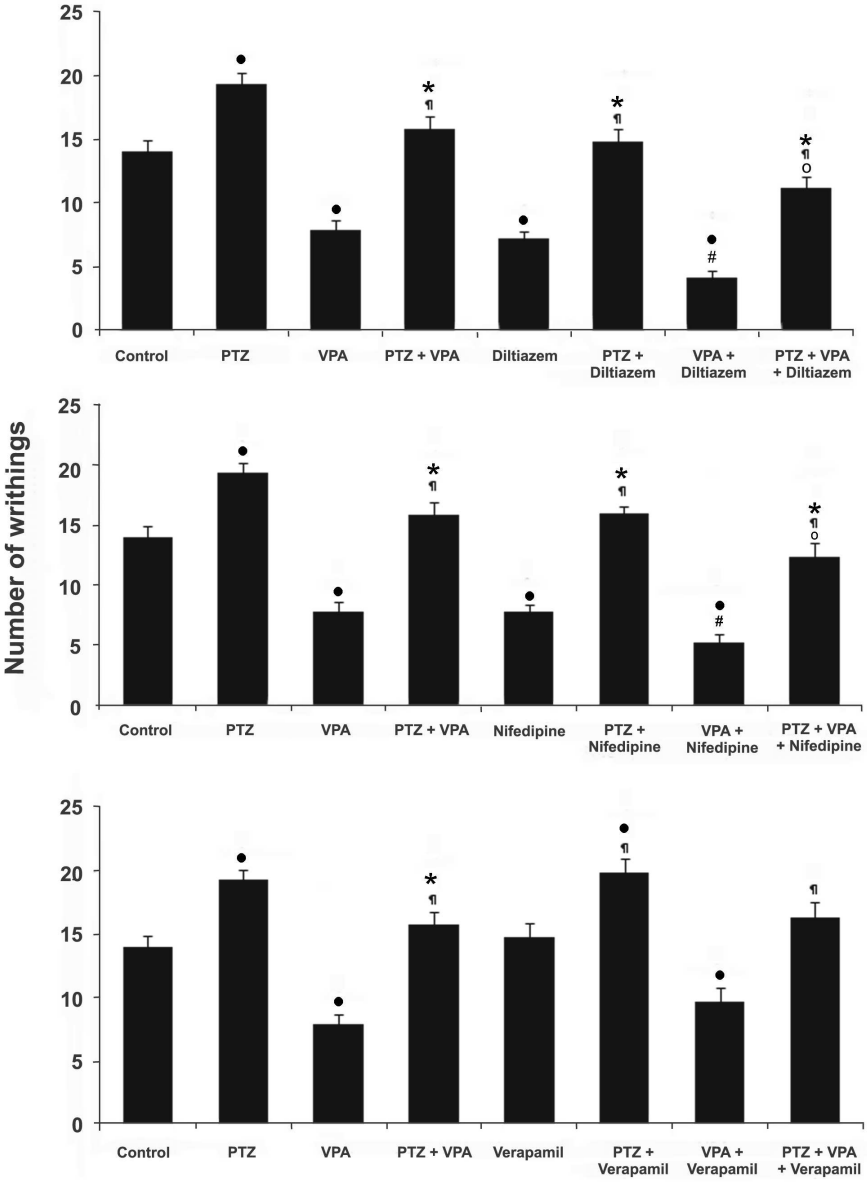


**Fig. 3.** Effects of daily *ip* administration of 20 mg/kg of diltiazem (upper panel), nifedipine (middle panel), or verapamil (lower panel) either alone or in combination with VPA (VPA, 200 mg/kg), on the number of writhings induced by acetic acid solution (0.8%; 0.1 ml/10 g, *ip*) in control as well as PTZ-kindled (35 mg/kg, *ip*, every other day, for a total of 11 injections) mice. Values are expressed as the mean ± SEM. All data were analyzed using ANOVA followed by Bonferroni *post-hoc* test; • $p \leq 0.05$ with respect to control, * $p \leq 0.05$ with respect to PTZ, # $p \leq 0.05$ with respect to VPA, ° $p \leq 0.05$ with respect to PTZ + VPA, ¶ $p \leq 0.05$ with respect to corresponding control. Each group consisted of 7 mice

parison with control group. On the other hand, control mice treated with VPA or the calcium channel blockers, diltiazem or nifedipine, but not verapamil, showed a significantly ($p < 0.05$) reduced number of writhings compared to untreated control group. Furthermore, treatment of kindled groups with valproic acid, diltiazem, or nifedipine significantly ($p < 0.05$) decreased the number of writhings as compared to PTZ-treated group to restore it to normal level. Conversely, administration of verapamil to kindled mice showed no significant difference from PTZ-treated group. In addition, the results indicated that control mice treated with VPA in combination with any of the three studied calcium channel blockers showed a significantly ($p < 0.05$) reduced number of writhings compared to untreated control mice. It is also observed that combining diltiazem or nifedipine, but not verapamil, with VPA significantly decreased the number of writhings observed in control mice when compared to valproic acid-treated control group. Comparable results were observed in kindled groups treated with the previous combination where a significant ($p < 0.05$) decreased number of writhings was detected as compared to untreated kindled group (Fig. 3).

## Discussion

This study describes, for the first time, the enhancing effect of calcium channel blockers on both the anticonvulsant and antinociceptive activities of VPA in a PTZ animal convulsion model. Seizures can arise when there is a disruption of mechanisms that normally create a balance between excitation and inhibition. Kindled seizures are widely accepted as an animal model of temporal lobe epilepsy, wherein repeated sub-threshold brain stimulation, electrical or chemical, leads to behavioral signs of tonic and clonic seizures [50]. This model of epilepsy has the advantages of both an epileptogenic and a spontaneous seizure model. Since spontaneity and recurrence of seizures are the basic features of human epilepsy, chronic models like kindling are advantageous over acute models [4].

Valporic acid and its salts, sodium or magnesium valproate, are effective antiepileptics with a broad spectrum of activity [3], however, there is a discrepancy in its effect in animal models. A study performed by Gasior et al. showed that VPA failed to reduce the sensitivity of kindled mice to the convulsive and lethal effects of PTZ [21]. Similarly, VPA did not modify the course of kindling induced by PTZ in the study carried out by Ilhan et al. [26]. In contrast, the current study showed that the administration of VPA induced protection against sub-convulsive doses of PTZ in kindled mice. Our results came in parallel with previous studies which indicated that VPA increased PTZ thresholds to different seizure types [24, 37]. The protective effect of VPA on PTZ-induced kindling is believed to be achieved through different neural mechanisms including inhibition of the voltage-dependent sodium channels, increased concentration of natural inhibitor GABA in CNS synapses, facilitation of GABAergic neurotransmission, reduced N-methyl-D-aspartate (NMDA)-receptor mediated glutamate excitation, increased serotonergic inhibition and attenuation of neurogenic inflammation [12, 28, 35, 36, 44, 57].

Calcium ion plays an important role in the pathogenesis of epilepsy. A calcium ion flux into the intracellular space represents the first stage of epileptic neuronal events [10] where an increased intracellular calcium level or enhanced calcium conductance can be observed during epileptic activity [54, 56]. In the present study, the calcium channel blockers, diltiazem, nifedipine or verapamil induced protection against sub-convulsive doses of PTZ in kindled mice, suggesting their beneficial role in reducing seizure severity. Our finding is in contrary with previous study reported by Czuczwar et al. [14], where none of these calcium channel blockers showed any protective effect against PTZ-induced convulsions. The use of different kindling strategies could explain the diverse results obtained in both experiments. The protection afforded by the three calcium channel blockers was comparable to the standard antiepileptic drug VPA. This finding is consistent with previous findings which demonstrated that epileptic depolarizations of neurons were found to be depressed by calcium ion channel blockers [11, 23, 46, 49]. In addition, co-administration of diltiazem with VPA showed a further significant reduction in kindling stage suggesting that the protective effect of VPA against epileptic seizures is enhanced by diltiazem.

Antiepileptic drugs display common antinociceptive and analgesic effects. Perception in general, including pain perception, is strongly influenced by the functional status of the brain [38, 39, 42]. Several reports indicated that epilepsy patients suffer from different types of pain [8, 15, 19, 60]. However, very few reports demonstrated the occurrence of neuropathic pain in epilepsy patients [43]. Neuropathic pain, whether of peripheral or central origin, is characterized by a neuronal hyperexcitability in damaged areas of the nervous system due to a series of molecular changes in the brain, the dorsal horn of the spinal cord, dorsal root ganglia, and the peripheral nociceptors [5, 53]. The data obtained in the current study demonstrated that kindling with PTZ significantly intensified the nociceptive effect induced either thermally or chemically. In contrast, Mareš and Rokyta have indicated that antinociception occurs following epileptic tonic-clonic seizures in experimental animal [41]. This discrepancy may be due to using different experimental design. On the other hand, the antinociceptive effect of VPA has been indicated in earlier studies in animal models and human [36, 57]. Our findings came in parallel with previously reported results where the administration of VPA to control mice as well as PTZ-treated mice showed significant antinociceptive effect in two experimental models of pain. The antinociceptive effect of VPA could be explained based on its previously mentioned neural mechanisms of action [35, 36, 44]. Similarly, other studies have used the hot plate to evaluate the antinociceptive effect of some antiepileptic drugs or their

derivatives [38, 39, 45]. Our study represents an additional evidence that antiepileptic agents could be useful in the management of acute nociceptive pain.

Recently, great progress has been made in understanding the cellular targets for anticonvulsant agents. In addition to blocking voltage-gated ion channels in the CNS (e.g., $Ca^{2+}$ channels), these agents can affect the same cellular targets in cell bodies of peripheral sensory neurons that convey nociceptive signals to the spinal cord [52]. Several types of calcium channels have been shown to be prominently involved in pain regulation [59]. A recent discovery showed that increases in calcium ion promote ischemia-induced neuronal injury [55]. This could explain the antinociceptive effect obtained in the current study following the administration of calcium channel blockers, diltiazem or nifedipine, to control mice or PTZ-treated mice in writhing test. The observed potentiating effect of concurrent administration of diltiazem or nifedipine on the antinociception exerted by VPA in control mice as well as PTZ-treated mice could be explained on the same bases. Our data confirm the previously reported antinociceptive effect of diltiazem and nifedipine [2, 31]. Verapamil failed to produce a similar antinociceptive effect. This could be due to the pharmacodynamic properties of this calcium channel antagonist [22, 32]. The observed antiepileptogenic effect of verapamil could be due to its antioxidant and neuroprotective activities [1, 30]. An alternative possibility is that verapamil, a known P-glycoprotein inhibitor [25], acted by facilitating the brain penetration of the concurrently administered VPA.

## Conclusions

It is concluded from the present study that the calcium channel blockers, diltiazem, nifedipine and verapamil decreased the kindling stage produced by PTZ to a comparable level of VPA. The antiepileptic drug, VPA, as well as diltiazem or nifedipine, but not verapamil, produced antinociception in two mouse models of pain. A novel finding of this study is that co-administration of diltiazem or nifedipine with VPA retained nociception to normal levels thus indicating the superiority of combined treatment to individual regimens. Further, the enhanced anticonvulsant effect observed up on combined therapy allows for lower doses with definitely less toxic concentrations of VPA to be therapeutically applied. If the results from this study could be extrapolated to clinical settings, the combination of calcium channel blockers with VPA might be beneficial for both epilepsy and pain relief in humans.

**Acknowledgments:**
The authors thank Sigma Pharmaceutical Industrial Co., Egypt, for the generous gift of diltiazem and verapamil. Nifedipine was kindly provided by Epico Pharmaceutical Industrial Co., Egypt.

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**Received:** May 17, 2011; **in the revised form:** October 1, 2011; **accepted:** November 21, 2011.