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Amiloride protects against pentylenetetrazole-induced kindling in mice

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1 This study was performed to investigate whether or not amiloride, a sodium–hydrogen exchanger (NHE) inhibitor, can protect against seizure development of pentylenetetrazole (PTZ)-induced kindling in mice.

2 Kindling was induced by once every 2 days treatment with PTZ (25 mg kg^{-1} i.p.) for 5 weeks. Challenge experiments were carried out after 15 or 30 days of last treatment with PTZ.

3 Administration of amiloride (2 h before PTZ, in doses of 0.65 and 1.3 mg kg^{-1}, p.o.) significantly prolonged the onset of kindling and reduced the incidence and severity of seizures in a dose-dependent manner. The effect of amiloride on the incidence of PTZ-induced seizures was evident even after 15 or 30 days of last treatment.

4 The results indicate a protective role for amiloride against PTZ-induced kindling in mice. The possibility of mediation of such effects by NHE inhibition is discussed.
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Abbreviations: NHE, sodium–hydrogen exchanger; PTZ, pentylenetetrazole

Introduction

The strategies for the development of antiepileptic drugs have heavily relied on the basic premise that epilepsies are due to an imbalance between excitatory and inhibitory transmission in the brain. Basic research in epilepsy is proceeding at a rapid pace and, with the advent of neurobiological techniques, there has been a tremendous increase in our knowledge on the possible molecular defects in epilepsies and a substantial number of molecular targets for antiepileptic drug development and screening have been identified. One of the most significant advancement is the concept of intraneuronal pH modulation and the potential of sodium–hydrogen exchanger (NHE) system in this regard (Whittingham *et al*., 1989; Bonnet *et al*., 2000). NHE is a ubiquitous transporter present in the plasma membranes of nearly all mammalian cells (Grinstein & Rothstein, 1986; Krapf & Alpern, 1993). The antiporter, by exchanging intracellular H^+ for extracellular Na^+, plays an essential role in a variety of cell functions, including pH regulation, volume homeostasis and cell growth (Grinstein & Rothstein, 1986; Krapf & Alpern, 1993). Various investigators have reported the *in vitro* efficacy of NHE inhibitors in suppressing epileptiform activity elicited by different pharmacological means (Sokolova *et al*., 1992; Bonnet *et al*., 2000). Surprisingly, our literature survey could not find any *in vivo* report that can corroborate earlier *in vitro* results. This prompted us to investigate the effect of amiloride, a NHE inhibitor, on electrically (increasing current electroshock) and chemically (pentylenetetrazole (PTZ)) induced seizures in mice, and we have recently reported a protective action for amiloride in these models (Ali *et al*., 2004). Although the predictive value of the maximal electroshock and PTZ models is still of use for antiepileptic drug development, models of other types of epilepsy should be added to these traditional models early during drug evaluation (Loscher, 1993).

Kindling is a model of epilepsy that has the advantages of both an epileptogenic and a spontaneous seizure model (McNamara, 1988). Since spontaneity and recurrence of seizures are the basic features of human epilepsy, chronic models like kindling are advantageous over acute models. There is little evidence, however, for the basic neuronal mechanisms underlying PTZ-kindled seizures. In the present study, we have investigated the effect of amiloride on PTZ-induced kindling in mice. In addition, challenge study was carried out 15 or 30 days after the end of repeated treatment.

Methods

Animals

Male albino Swiss strain mice weighing 18–30 g were used. Animals were housed in groups of 5–10 per cage and maintained at 20–30°C and 50–55% humidity in a natural light and dark cycle, with free access to food and water. The experiments were performed during the light cycle in awake, freely moving animals. Animals were procured from the central animal house, Jamia Hamdard, New Delhi. The animals were brought to the laboratory at least 2 h before use for acclimatization to the new premises. Only active and apparently healthy animals with no visible lesions or gross abnormalities were selected for experiments.

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The project was undertaken with prior approval from the University Animal Ethics Committee. Utmost care was taken to ensure that animals were treated in the most humane and ethically acceptable manner.

Induction of kindling and observational parameters

Kindling was induced according to the method of Sarro *et al.* (2000). Four groups of mice (20 in each group) were administered daily with vehicle, diazepam 3 mg kg^{-1}, amiloride 0.65 and 1.3 mg kg^{-1}, p.o. They were injected with a subconvulsant dose of PTZ (25 mg kg^{-1}, i.p.) once every 2 days for 5 weeks (2 h after vehicle or amiloride and 1.5 h after diazepam treatment) and the occurrence of epileptic signs was observed. One separate group of mice received vehicle only throughout the study and served as control group. The intensity of seizure response was scored as: 0 = no response, 1 = mouth and facial jerks, 2 = nodding or myoclonic body jerks, 3 = forelimb clonus, 4 = rearing, falling down (loss of postural control), hindlimb clonus and forelimb tonus, 5 = tonic extension of hindlimb, status epilepticus and/or death. Animals were observed for 30 min after PTZ injection. The maximum response was recorded in each animal. When the animal had a seizure score of 4 on three consecutive administrations, it was defined as being kindled and its treatment was discontinued. The mice in each group were also observed for latency to the onset of kindled seizures. The proportion of animals kindled at the end of the study was also recorded.

To study the effect of amiloride on kindled mice, four groups of kindled mice (six in each group) were treated with vehicle + PTZ (25 mg kg^{-1} i.p.), diazepam (3 mg kg^{-1}) + PTZ (25 mg kg^{-1} i.p.), amiloride (0.65 mg kg^{-1}) + PTZ (25 mg kg^{-1} i.p.), amiloride (1.3 mg kg^{-1}) + PTZ (25 mg kg^{-1} i.p.). Seizure severity was recorded according to the scale shown above.

Challenge with PTZ

PTZ (25 mg kg^{-1} i.p.) was administered 15 and 30 days after the end of chronic treatments (to same animals) and animals were observed for 30 min in order to determine the occurrence of clonic seizures. The experiment was carried out using five groups, each containing 10 mice: group I chronically treated with vehicle, group II kindled with vehicle + PTZ, group III diazepam (3 mg kg^{-1}) + PTZ, group IV amiloride (0.65 mg kg^{-1}) + PTZ, group V amiloride (1.3 mg kg^{-1}) + PTZ.

Drugs

The studies utilized the following drugs and chemicals: amiloride (Micro Nova Pharmaceuticals, India); PTZ (Sigma, U.S.A.); diazepam (Ranbaxy Labs, India).

The amiloride dose was calculated from the equivalent absolute human dose using the surface area ratio of mouse to man (Ali *et al.*, 2004). Diazepam dose was based on earlier published reports (Dollery, 1999). Amiloride and diazepam were dissolved in distilled water to desired concentrations, whereas PTZ was dissolved in normal saline. All drugs were given in a volume of 10 ml kg^{-1}. All observations were made 2 h after amiloride and 1.5 h after diazepam treatment that was observed as the time of peak anticonvulsant effect (Ali *et al.*, 2004) in our lab. Each mouse received only one type of treatment and test and was not reused.

Statistical analysis

Seizure severity scores were compared using Kruskal–Wallis one-way analysis of variance on ranks followed by multiple comparison tests. For incidence %, Fisher's exact probability test was used. *P*-values <0.05 were considered significant.

Results

Effect of amiloride on the development of PTZ kindling

Repeated treatment with PTZ at a subconvulsant dose (25 mg kg^{-1} i.p.), three times a week, induced chemical kindling (Figure 2).

Animals treated with diazepam (3 mg kg^{-1}) exhibited a significant reduction in incidence ($P<0.05$ using Fisher's exact probability test) (Figure 1) and severity of seizures ($P<0.05$ using Kruskal–Wallis analysis by ranks) (Figure 2). Pretreatment with amiloride (0.65 and 1.3 mg kg^{-1}, p.o., daily) reduced both the incidence ($P<0.05$) (Figure 1) as well as severity of seizures ($P<0.05$) (Figure 2) occurring during the repeated treatments with PTZ in a dose-dependent manner. In addition, amiloride at a dose of 1.3 mg kg^{-1} (but not at 0.65 mg kg^{-1}) significantly increased the latency to first kindled seizures (28 days as compared to 14 days in control group). Further, amiloride did not show any observable side effect during the course of treatment.

Effect of amiloride treatment on seizure severity in kindled mice

Administration of a previously subconvulsive dose (25 mg kg^{-1}, i.p.) of PTZ in kindled mice showed a mean seizure score of 4.2 (Figure 3). Diazepam (3 mg kg^{-1}) exhibited significant reduction ($P<0.01$) in seizure severity as compared

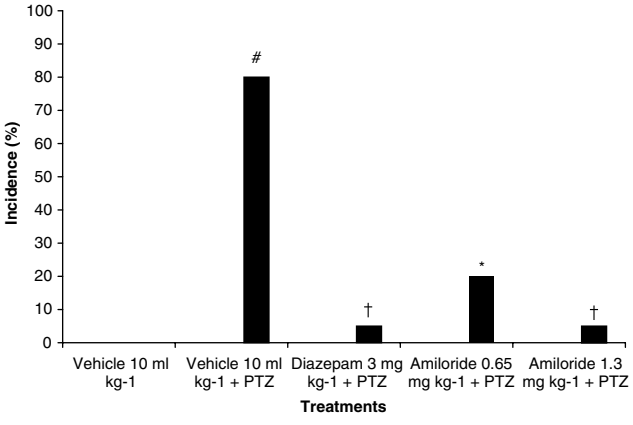

Figure 1 Effect of amiloride treatment on the incidence of animals kindled following repeated treatment with a subconvulsant dose of PTZ (25 mg kg^{-1}, i.p.). Vehicle, diazepam and amiloride were administered daily, while PTZ (25 mg kg^{-1} i.p.) was given once every 2 days for 5 weeks. $^{*}P<0.05$; $^{\dagger}P<0.01$ vs vehicle + PTZ; $^{\#}P<0.01$ vs vehicle, using Fisher's exact probability test. The number of animals in each group was 20.

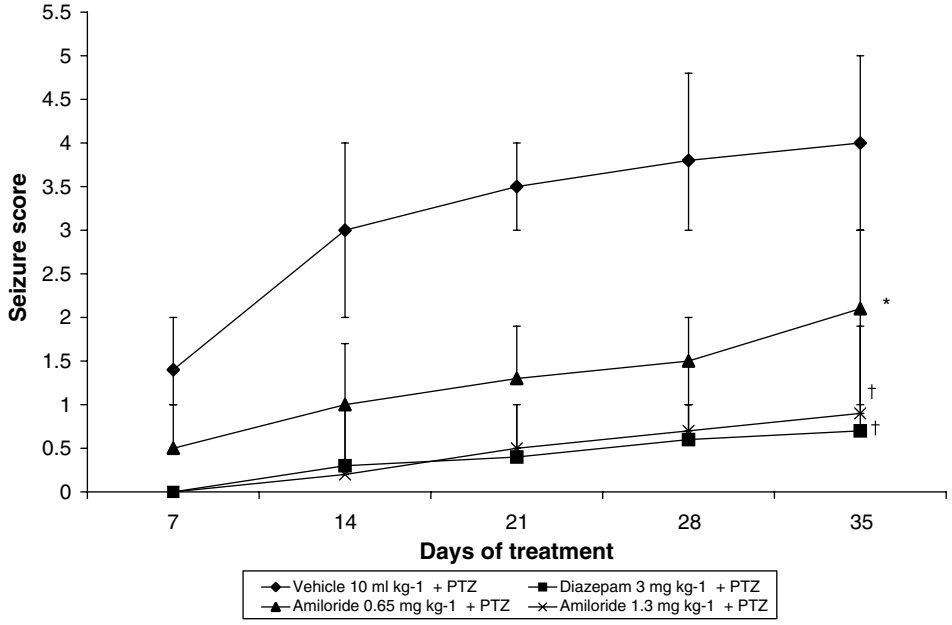

Figure 2 Effect of amiloride on seizure severity during induction of kindling by PTZ in mice. Vehicle, diazepam and amiloride were administered daily, while PTZ (25 mg kg^{-1} i.p.) was given once every 2 days for 5 weeks. Data are presented as median seizure score with upper and lower quartiles. $^{*}P<0.05$; $^{\dagger}P<0.01$ vs vehicle + PTZ, by Kruskall–Wallis analysis by ranks (at the end of 35 days). The number of animals per group was 20.

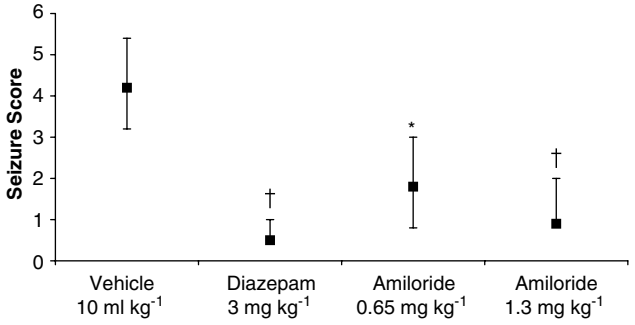

Figure 3 Effect of amiloride on seizure severity in PTZ-kindled mice. Data are presented as median seizure score with upper and lower quartiles. Significant differences are denoted by $^{*}P<0.05$; $^{\dagger}P<0.01$ vs vehicle, using Kruskal–Wallis analysis by ranks. The number of animals per group was 6.

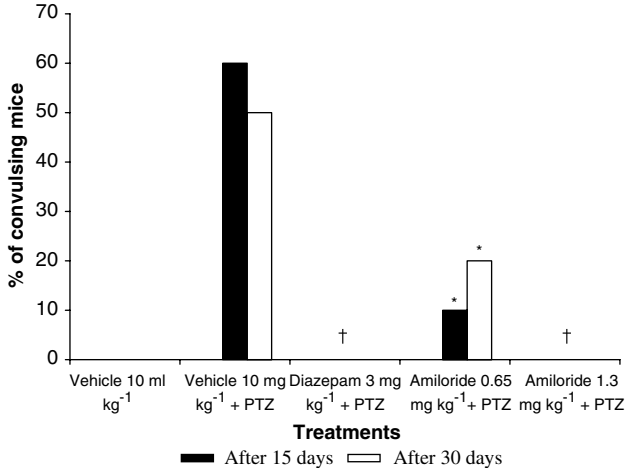

Figure 4 Effects of challenge with PTZ on mice receiving repeatedly vehicle, vehicle + PTZ, diazepam (3 mg kg^{-1}) + PTZ, amiloride (0.65 mg kg^{-1}) + PTZ or amiloride (1.3 mg kg^{-1}) + PTZ for 5 weeks. (■) after 15 days; (□) after 30 days. Animals were challenged with PTZ (25 mg kg^{-1}, i.p.) 15 or 30 days after the end of repeated treatment and occurrence of clonic seizures was observed. Significant differences are denoted by $^{*}P<0.05$; $^{\dagger}P<0.01$ vs the vehicle + PTZ group using Fisher's exact probability test. The number of animals per group was 10.

to the vehicle-treated group. Amiloride treatment (0.65 or 1.3 mg kg^{-1} p.o.) reduced the seizure severity of kindled mice in a dose-dependent manner ($P<0.05$ using Kruskal–Wallis analysis by ranks).

Challenge with PTZ

As shown in Figure 4, the challenge experiment carried out with PTZ (25 mg kg^{-1}, i.p.) 15 days after the end of repeated treatment demonstrated that PTZ elicited clonic seizures in six out of 10 mice receiving vehicle + PTZ, in one out of 10 mice receiving amiloride 0.65 mg + PTZ, but no clonic seizures were observed in 10 mice receiving amiloride 1.3 mg + PTZ. In addition, PTZ challenge after 30 days of repeated treatment exhibited clonic seizures in five out of 10 mice treated with vehicle + PTZ (25 mg kg^{-1}, i.p.) and in two out of 10 mice receiving amiloride 0.65 mg kg^{-1} + PTZ, but not in animals which were treated with amiloride (1.3 mg kg^{-1}) + PTZ (Figure 4). Similarly, animals repeatedly treated with diazepam (3 mg kg^{-1}) did not show clonic seizures after PTZ challenge (15 or 30 days).

Discussion

Chemical kindling is the development of seizures following repeated administration of a convulsant substance at a dose

that is insufficient to produce convulsions following a single administration (Lewin *et al*., 1989). Kindling produced by PTZ in mice has characteristics very similar to those described for electrical kindling, that is, the response to PTZ is proportional to the stimulus intensity. The PTZ kindling model (Mason & Cooper, 1972) is generally used as a laboratory model of human partial complex epilepsy (Kupferberg, 2001). The present study uncovered potent anticonvulsant and anti-epileptogenic properties of amiloride against PTZ-kindled seizures in mice.

Recent studies indicate that NHE isoforms are differentially regulated in certain pathophysiological states. The antiporter is present in most vertebrate cells and is the predominant mechanism for cytoplasmic pH regulation (Lin *et al*., 1996). It is well known that pH changes modulate neuronal activity and *vice versa* (Roos & Boron, 1981; Aram & Lodge, 1987; Chesler & Kaila, 1992). Neurones are reported to regulate an acidified pH by H^+ buffering, metabolism and activation of transmembrane acid extruders (Roos & Boron, 1981; Chesler, 1990; Bonnet *et al*., 1997). For the latter, distinct receptor-integrated ionophores (Chesler, 1990; Chesler & Kaila, 1992) and antiporters (e.g. Na^+-dependent Cl/HCO_3^-; Na^+/H^+) have been described (Chesler, 1990; Lin *et al*., 1996; Wakabayashi *et al*., 1997). Among the Na^+/H^+ exchangers, especially subtypes 1 and 4 are highly abundant within pyramidal cells of the hippocampus (Lin *et al*., 1996; Ma & Haddad, 1997), the latter being an important region for epileptic activity. Amiloride is known to block these subtypes in the brain (Lin *et al*., 1996; Wakabayashi *et al*., 1997). Interestingly, a mutation in gene coding for NHE1 resulted in a spontaneous mutant mouse exhibiting slow wave epilepsy, with a neurological syndrome including ataxia and a unique epilepsy phenotype consisting of $3\,s^{-1}$ absence and tonic–clonic seizures (Cox *et al*., 1997).

In the present study, amiloride suppressed development of kindled seizures in mice. Further, it protected against a subconvulsant dose of PTZ in kindled mice, suggesting that it not only inhibits the development of epilepsy (epileptogenesis) but also seizure severity even when the disease state was fully developed. The protection afforded by amiloride ($1.3\,mg\,kg^{-1}$) was comparable to the standard antiepileptic drug diazepam. The protective effect of diazepam on PTZ kindling is well established (Schwark & Haluska, 1987) and is known to occur *via* the interaction either with the benzodiazepine-binding site at the GABA-benzodiazepine receptor ionophores complex ($GABA_A$ receptor). The protection afforded by amiloride, in our study, is consistent with the various *in vitro* reports where NHE inhibitors reduced epileptiform activity in hippocampal slices elicited by different pharmacological strategies such as bicuculline, caffeine or zero magnesium (Bonnet *et al*., 2000). In addition, Sokolova *et al*. (1992) reported an inhibitory effect of amiloride on epileptic activity in the rat temporal cortex slices. Recently, we have reported a protective action of amiloride in *in vivo* seizure models in rodents including increasing current electroshock seizure threshold and PTZ tests (Ali *et al*., 2004). It has been suggested that inhibition of NHE results in sustained intracellular acidification (Bonnet *et al*., 2000). The latter is known to terminate epileptiform discharges (Xiong *et al*., 2000) and to attenuate glutamate neurotoxicity as well as Ca^{2+}-mediated neuronal injury observed in epileptics (Giffard *et al*., 1990; Puka & Lehmann, 1994). Though intracellular acidification appears to be a primary mechanism for the anticonvulsant action of amiloride, other effects may not be ruled out, including inhibition of transmembrane low-threshold Ca^{2+} channels (Higashima *et al*., 1998) and inhibition of voltage-gated Na^+ channels (Velly *et al*., 1988), the latter being an important mechanism for many of the established antiepileptic drugs.

While there is little evidence for the basic neuronal mechanisms underlying PTZ-kindled seizures, Schroder *et al*. (1998) showed an increase in glutamate binding and glutamate concentrations in the hippocampus following PTZ kindling. Other investigators have reported an enhanced activity of glutamatergic system and an increased brain concentration of NMDA following kindling (Schroder *et al*., 1993; Walsh *et al*., 1999). It is presumed that an enhancement of glutamate receptor density may be crucially related to an increased susceptibility of target neurons to L-glutamate in the course of development and persistence of both the electrically and chemically induced kindling phenomenon in mice (Getova *et al*., 1998). The protection afforded by amiloride, in the present model, thus, could be related to an indirect effect on the glutamatergic system *via* intracellular acidification produced by inhibition of NHE (Giffard *et al*., 1990; Puka & Lehmann, 1994). The anticonvulsant action of amiloride in the challenge study further confirms its inhibitory action on development of PTZ-kindled seizures, as clonic seizures were observed in the vehicle + PTZ group (but not in the amiloride $1.3\,mg\,kg^{-1}$-treated group) even after 15 or 30 days of last treatment.

While further studies are required to establish the exact basis for antiseizure activity of amiloride, the present study clearly demonstrates its efficacy against yet another model of epilepsy. Since seizures observed following PTZ kindling are considered a drug-resistant model of epilepsy (Loscher, 1993), inhibition of NHE may represent a possible therapeutic strategy for preventing the generation of some of the resistant forms of epilepsy. Overall, the results of the present study add to the accumulating evidence suggestive of the therapeutic potential of amiloride in the treatment of some types of epilepsies.

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