Brain Research Bulletin 90 (2013) 52–57

Contents lists available at SciVerse ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

Probing the role of the sodium/calcium exchanger in pentylenetetrazole-induced generalized seizures in rats

Prosper N'Gouemo*

Department of Pediatrics, Bldg. D, Room 285, Georgetown University Medical Center, 3900 Reservoir Rd, NW, Washington, DC 20057, USA

ARTICLE INFO

Article history:
Received 3 September 2012
Accepted 10 September 2012
Available online 17 September 2012

Keywords:
KB-7943
SN-6
Anticonvulsant
Hyperexcitability
Clonic seizures
Tonic–clonic seizures

ABSTRACT

The Na^+/Ca^{2+} exchanger (NCX) is thought to play an important role in the pathogenesis of pentylenetetrazole (PTZ)-induced tonic flexion in mice. Here, I investigated the expression of PTZ-induced generalized clonic and tonic–clonic seizures in rats, using two potent NCX reverse mode inhibitors, KB-R7943 and SN-6 for NCX subtypes 3 (NCX3) and 1 (NCX1), respectively. Pretreatment with KB-R7943 (3, 10, and 30 mg/kg; p.o.) significantly reduced the expression of PTZ-induced generalized seizures with clonic and tonic–clonic components in 12–62% and 25–62% of the treated animals, respectively. In the remaining animals that exhibited seizures, KB-R7943 (3 mg/kg; p.o.) pretreatment significantly delayed the onset of the first seizure episode and reduced the seizure severity. Following pretreatment with SN-6 (0.3, 1, 3, 10, and 30 mg/kg; p.o.), clonic and tonic–clonic PTZ-induced generalized seizures were reduced in 25–50% and 38–63% of treated animals, respectively. SN-6 (0.3, 1, and 3 mg/kg; p.o.) also significantly reduced PTZ-induced seizure severity scores, but did not alter seizure latencies. KB-R7943 (3 and 30 mg/kg; p.o.) or SN-6 (3 and 30 mg/kg; p.o.) administration potentiated the sub-anticonvulsant dose of diazepam (2.5 mg/kg; i.p.) that suppresses clonic and tonic–clonic PTZ-induced seizures. These findings suggested that Ca^{2+} influx via the NCX in reverse mode contributes to a neuronal hyperexcitability that leads to clonic and tonic–clonic generalized seizures and that the NCX1 and NCX3 isoforms may serve as novel molecular targets for seizure suppression.

1. Introduction

The Na^+/Ca^{2+} exchanger (NCX) is a bidirectional membrane ion transporter that couples the counter-transport of Na^+ and Ca^{2+} to regulate the levels of intracellular Ca^{2+} in various cell preparations (Blaustein and Lederer, 1999; Annunziato et al., 2004). Under physiological conditions when intracellular Ca^{2+} levels rise, the NCX couples the export of a Ca^{2+} ion to the import of three Na^+ ions; this is known as the "forward" mode of NCX activity. However, when intracellular Na^+ levels rise or strong membrane depolarization occurs, the exchanger reverses, exporting three Na^+ ions for each imported Ca^{2+} ion; this is referred to as the "reverse" mode of NCX activity (Blaustein and Lederer, 1999; Annunziato et al., 2004). Elevated intracellular Ca^{2+} and altered Ca^{2+} homeostasis have been implicated in the pathogenesis of epilepsy. Thus, massive Ca^{2+} entry into the cell following activation of the NCX in reverse mode can disturb Ca^{2+} homeostasis, resulting in neuronal hyperexcitability that can lead to seizures. Three different isoforms of the NCX (NCX1, NCX2 and NCX3) have been characterized, cloned and detected in various tissues, including the central nervous system (Philipson and Nicoll, 2000; Quednau et al., 1997; Papa et al., 2003; Lytton, 2007). The NCX has been implicated in the pathophysiology of various neurological conditions, including Alzheimer's disease (Bi et al., 2012; Sokolow et al., 2011), ischemia (Pignataro et al., 2004; Lee et al., 2005; Boscia et al., 2000), hypoxia (Secondo et al., 2007) and Parkinson's disease (Ago et al., 2011). Although the role of the NCX in the pathogenesis of seizures and epilepsy remains poorly understood, we have reported that inhibition of Ca^{2+} influx via NCX activity in the reverse mode reduced the incidence of pilocarpine-induced limbic seizures and status epilepticus (Martinez and N'Gouemo, 2010). The NCX also plays an important role in the pathogenesis of generalized seizures because genetic deletion of the NCX1 isoform suppressed the tonic flexion component of pentylenetrazole (PTZ)-induced generalized seizures in mice (Saito et al., 2009). The NCX-3 isoform is also implicated in the pathogenesis of tonic–clonic seizures in Mongolian gerbils, a model of inherited epilepsy (Park et al., 2011). It remains unknown whether NCX in rats contributes to the pathogenesis of clonic and tonic–clonic components of PTZ-induced generalized seizures. Here, I sought to determine the extent to which pharmacological blockade of reverse mode NCX activity may alter the expression and severity of PTZ-induced generalized seizures in rats.

Abbreviations: DZP, diazepam; NCX, sodium/calcium exchanger; PTZ, pentylenetetrazole.

* Tel.: +1 202 687 8464; fax: +1 202 444 7161.
E-mail address: pn@georgetown.edu

0361-9230/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.brainresbull.2012.09.007

2. Materials and methods

Sprague–Dawley rats (male, 150–200 g, Taconic, Germantown, NY) were used in accordance with the NIH guidelines for use and care of laboratory animals and the Georgetown Animal Care and Use Committee approved all experiments. I minimized the number of animals used and their discomfort. Two doses of PTZ (50 or 60 mg/kg; Sigma Chemicals, St. Louis, MO) were tested to determine a minimum effective dose that was used in subsequent experiments. PTZ was dissolved in 0.9% saline and intraperitoneally (i.p.) injected to induce seizures. After PTZ injections animals were placed in clear plexiglass boxes for 60 min to monitor for the occurrence of seizure activity. Convulsive seizure behavior was classified as follows (Luttjohann et al., 2009, modified): stage 0, no response; stage 1, myoclonic jerks; stage 2, myoclonus (i.e., clonic seizures while the animal is lying on its belly); stage 3, bilateral forelimb clonic seizures without rearing; stage 4, forelimb clonic seizures and rearing; stage 5, tonic–clonic seizures. The acute PTZ model was chosen because generalized seizures induced by this method exhibits a gradual development over a period of approximately 2 min compared to seizures induced by electrical stimulation, which are characterized by an abrupt onset. To evaluate the role of the NCX in the development of PTZ-induced generalized seizures, I antagonized reverse mode NCX activity using two inhibitors, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate (KB-R7943; Tocris Bioscience, Ellisville, MO) and the structurally related (2-[[4-[(4-nitrophenyl)methoxy]phenyl]methyl]-4-thiazoli dinecarboxylic acid ethyl ester (SN-6; Tocris Bioscience, Ellisville, MO). Animals were randomly separated into groups of 8 and those that only received the vehicle were used as controls. KB-R7943 (1, 3, 10, and 30 mg/kg) and SN-6 (0.3, 1, 3, 10, and 30 mg/kg) were dissolved in sterile water, filtered and administered 90 min before PTZ injections. KB-R7943 and SN-6 were given orally (p.o.) by gastric intubation in a volume of 0.2 ml/100 g body weight using an 18-gauge stainless steel feeding needle with a round tip (ball diameter 3 mm). The initial dose of 0.3 or 1 mg/kg was chosen based on published in vivo pharmacological studies and preliminary data (Martinez and N'Gouemo, 2010; Blokhin et al., 2008). The 90 min timepoint was the most effective pretreatment window on seizure activity in our previous study (Martinez and N'Gouemo, 2010). In another series of experiments, I compared the anticonvulsant efficacy of KB-R7943 and SN-6 to diazepam (DZP), a clinically used anticonvulsant. DZP (2.5 and 5 mg/kg in 0.9% saline) was intraperitoneally injected into rats 90 min before the PTZ (60 mg/kg; i.p.) challenge. I also evaluated the extent to which a combination of either KB-R7943 (3 mg/kg; p.o.) or SN-6 (3 mg/kg; p.o.) with a sub-anticonvulsant dose of DZP (2.5 mg/kg; i.p.), administered 90 min before the PTZ (60 mg/kg; i.p.) challenge, prevented the occurrence of generalized seizures. At the end of the experiment, animals were euthanized with a lethal dose of Nembutal (100 mg/kg, i.p.). Following a given pharmacological pretreatment and PTZ challenge, animals that did not display class 1 seizures within the 60 min observation period were considered to be protected from seizure. For each group of animals, the incidences of clonic and tonic–clonic components of PTZ-induced generalized seizures were also recorded. The time intervals from the end of PTZ injections to the appearance of the first seizure episode were recorded, and this period was referred to as seizure latency. For each animal, the seizure severity score was also recorded.

Analysis of the incidences of PTZ-induced generalized clonic and tonic–clonic seizures was performed using a Chi-squared (χ^2) test. The seizure latencies were analyzed using a one-way ANOVA with a Dunn's post hoc test for multiple comparisons. Before using an ANOVA, the data were subjected to a normality test (i.e., the Shapiro–Wilk test) and a test for homogeneity of variances (i.e., the Levene's test). Comparison of seizure severity scores was assessed with the Kruskal–Wallis rank test and the Dunn's post hoc test. The cut-off for statistical significance was $p < 0.05$. All data are presented as the mean ± S.E.M.

3. Results

The incidence of PTZ-induced seizures was first evaluated using two doses of PTZ (50 and 60 mg/kg; i.p.). At the 50 mg/kg dose, 25% of the animals ($n = 4$) did not exhibit seizures, but clonic and tonic–clonic PTZ-induced seizures were observed in 75% and 50% of the animals, respectively. In contrast, all animals ($n = 4$) treated with PTZ at the dose of 60 mg/kg exhibited generalized seizures. Therefore, for subsequent experiments PTZ was used at a dose of 60 mg/kg.

In the control group, the incidences of clonic and tonic–clonic PTZ-induced generalized seizures were 100% (8/8) and 87.5% (7/8), respectively. The latency to the first seizure episode was 111 ± 14 s ($n = 8$) and the seizure severity was 4.8 ± 0.1 ($n = 8$). KB-R7943 pretreatment significantly reduced ($\chi^2 = 85$, $p = 0.0001$) the incidence of clonic PTZ-induced generalized seizures. Compared to the control group, this reduction was observed in animals challenged with, 3 mg/kg (5/8 ($\chi^2 = 43$, $p = 0.000$); Fig. 1A), 10 mg/kg (7/8 ($\chi^2 = 11$, $p = 0.001$); Fig. 1A) and 30 mg/kg of PTZ (7/8 ($\chi^2 = 11$, $p = 0.001$); Fig. 1A) but not at 1 mg/kg (8/8; Fig. 1A). Similarly, the incidence of tonic–clonic PTZ-induced generalized seizures was reduced following KB-R7943 pretreatment ($\chi^2 = 85$, $p = 0.0001$). This reduction was observed following treatment with KB-R7943 at doses, 3 mg/kg (3/8 ($\chi^2 = 57$, $p = 0.0001$); Fig. 1B), 10 mg/kg (6/8 ($\chi^2 = 5$, $p = 0.03$); Fig. 1B) and 30 mg/kg (6/8 ($\chi^2 = 5$, $p = 0.03$); Fig. 1B) when compared to the control group (6/8; Fig. 1B). In the remaining animals that exhibited PTZ-induced generalized seizures, KB-R7943 pretreatment significantly delayed the onset of the first seizure episode ($F = 9$, $p = 0.0001$; Fig. 1C). This delay was observed for a KB-R7943 dose of 3 mg/kg (157 ± 12 s, $n = 5$; Fig. 1C), but not 1 mg/kg (138 ± 12 s, $n = 8$), 10 mg/kg (91 ± 11 s, $n = 7$) or 30 mg/kg (71 ± 4 s, $n = 7$), when compared to the control group (111 ± 14 s, $n = 8$). Pretreatment with KB-R7943 also significantly reduced the seizure severity score ($H = 9.6$; $p = 0.05$). This effect was observed at a dose of 3 mg/kg (2.2 ± 0.8, $n = 8$, Fig. 1D), but not 1 mg/kg (4.8 ± 0.3, $n = 8$), 10 mg/kg (4 ± 0.6, $n = 7$) and 30 mg/kg (4.0 ± 0.5, $n = 7$) compared to the control group (4.9 ± 01, $n = 8$; Fig. 1C).

To verify the results of KB-R7943 I evaluated the effects of SN-6 on PTZ-induced generalized seizures. SN-6 pretreatment significantly reduced the incidence of PTZ-induced generalized clonic seizures ($\chi^2 = 127$; $p = 0.0001$). This effect was observed for the doses above 0.3 mg/kg (1 mg/kg, 7/8 ($\chi^2 = 11$, $p = 0.001$); 3 mg/kg, 4/8 ($\chi^2 = 64$, $p = 0.0001$); 10 mg/kg, 6/8 ($\chi^2 = 26$, $p = 0.0001$); 30 mg/kg, 8/8 ($\chi^2 = 11$, $p = 0.001$); Fig. 2A) compared to the control group (8/8). SN-6 pretreatment also markedly reduced the incidence of PTZ-induced tonic–clonic seizures ($\chi^2 = 108$, $p = 0.0001$). This effect was also observed at all doses tested (1 mg/kg, 3/8 ($\chi^2 = 87$, $p = 0.0001$); 3 mg/kg, 3/8 ($\chi^2 = 87$, $p = 0.0001$); 10 mg/kg, 4/8 ($\chi^2 = 64$, $p = 0.0001$); 30 mg/kg, 5/8 ($\chi^2 = 30$, $p = 0.0001$); Fig. 2B), compared to the control group (8/8). In the remaining animals that exhibited seizures, SN-6 pretreatment did not significantly increase the seizure latency (148 ± 27 s, $n = 8$) at the dose of 0.3 mg/kg compared to controls (111 ± 14 s, $n = 8$; Fig. 2C). No changes in seizure latencies were observed with doses above 0.3 mg/kg (1 mg/kg, 89 ± 7 s, $n = 8$; 3 mg/kg, 97 ± 10 s, $n = 4$; 10 mg/kg, 112 ± 19 s, $n = 6$; 30 mg/kg, 83 ± 11 s, $n = 7$, compared to controls, 111 ± 14 s, $n = 8$). Nevertheless, the seizure severity scores were significantly reduced following SN-6 pretreatment ($H = 9.6$, $p = 0.05$). This effect was observed at a dose of 3 mg/kg (2 ± 0.8, $n = 8$; Fig. 2D) but not 1 mg/kg (3.4 ± 0.6, $n = 7$), 10 mg/kg (3.3 ± 0.8, $n = 6$) and 30 mg/kg (4 ± 0.8, $n = 8$) compared to the control group (4.9 ± 0.1, $n = 8$; Fig. 2D).

Diazepam, a clinically used anticonvulsant was used to validate the PTZ model and compare its efficacy to that of KB-R7943 and SN-6. Pretreatment with DZP, at the dose of 2.5 mg/kg, reduced the incidence of clonic (6/8 ($\chi^2 = 26.3$, $p = 0.0001$); Fig. 3A) and tonic–clonic (5/8 ($\chi^2 = 14$, $p = 0.0001$); Fig. 3B) seizures compared to the control group, but failed to alter the seizure latency (90 ± 12 s, $n = 4$; controls, 111 ± 14 s, $n = 8$; Fig. 3C) and seizure severity score (3.2 ± 0.8, $n = 8$; controls, 4.8 ± 0.1, $n = 8$; Fig. 3D). At the dose of 5 mg/kg, diazepam completely suppressed the occurrence of clonic and tonic–clonic component of PTZ-induced generalized seizures (Fig. 3A and B). I also evaluate the extent to which a combination of KB-R7943 (3 mg/kg; p.o.) and DZP (2.5 mg/kg; i.p.), as well as SN-6 (3 mg/kg; p.o.) and DZP (2.5 mg/kg; i.p.) affects the development of PTZ-induced generalized seizures. Co-administration of KB-R7943 (3 mg/kg; p.o.) and DZP (2.5 mg/kg; i.p.) significantly reduced the incidence of clonic seizures ($\chi^2 = 67$, $p = 0.0001$; Fig. 3A), nearly suppressed the occurrence of tonic–clonic seizures ($\chi^2 = 118$, $p = 0.0001$; Fig. 3B), non-significantly increased the seizure latency (Fig. 3C) and significantly reduced the seizure severity ($H = 9$, $p = 0.01$; Fig. 3D), compared to controls. Co-administration of KB-R7943 (3 mg/kg; p.o.) and DZP (2.5 mg/kg; i.p.) also significantly reduced the incidence of clonic seizures ($\chi^2 = 12$, $p = 0.0001$; Fig. 3A) and tonic–clonic seizures ($\chi^2 = 51$, $p = 0.0001$; Fig. 3B),