Epilepsy Research (2014) **108**, 1705—1710

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

Long-term decrease in Na^+,K^+-ATPase activity after pilocarpine-induced status epilepticus is associated with nitration of its alpha subunit

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Received 5 June 2014; received in revised form 20 August 2014; accepted 21 September 2014
Available online 28 September 2014

KEYWORDS
Epilepsy;
Pilocarpine;
Sodium pump;
Nitrotyrosine

Abstract Temporal lobe epilepsy (TLE) is the most common type of epilepsy with about one third of TLE patients being refractory to antiepileptic drugs. Knowledge about the mechanisms underlying seizure activity is fundamental to the discovery of new drug targets. Brain Na^+,K^+-ATPase activity contributes to the maintenance of the electrochemical gradients underlying neuronal resting and action potentials as well as the uptake and release of neurotransmitters. In the present study we tested the hypothesis that decreased Na^+,K^+-ATPase activity is associated with changes in the alpha subunit phosphorylation and/or redox state. Activity of Na^+,K^+-ATPase decreased in the hippocampus of C57BL/6 mice 60 days after pilocarpine-induced status epilepticus (SE). In addition, the Michaelis—Menten constant for ATP of $\alpha 2/3$ isoforms increased at the same time point. Nitration of the α subunit may underlie decreased Na^+,K^+-ATPase activity,

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http://dx.doi.org/10.1016/j.eplepsyres.2014.09.025

however no changes in expression or phosphorylation state at Ser^{943} were found. Further studies are necessary define the potential of nitrated Na^+,K^+-ATPase as a new therapeutic target for seizure disorders.

Introduction

Epilepsy is one of the most common serious neurological condition and a major worldwide public health problem (Schmidt and Sillanpaa, 2012). Accordingly, the World Health Organization (2006) estimates that epilepsy may contribute around 1% of the global total of days lost due to disease, and about 7.9% of the disability-adjusted life years caused by neurological disorders. Temporal lobe epilepsy (TLE) is the most common type of human epilepsy, and is usually triggered by an initial precipitating injury such as *status epilepticus* (*SE*), head trauma, childhood febrile seizures, or hypoxia (Acharya et al., 2008). Importantly, pharmacological treatment of TLE is completely effective in controlling the seizures in fewer than 20% of patients (Schmidt and Sillanpaa, 2012). In this context, knowledge about the mechanisms underlying seizure activity is fundamental to discovery of new drug targets.

Compelling evidence indicates that Na^+,K^+-ATPase plays a role in several neurological disorders, including seizure activity, and therefore may constitute a potential new target for treatment of these conditions (Aperia, 2007; Benarroch, 2011). In the brain, the activity Na^+,K^+-ATPase contributes crucial for maintaining the electrochemical gradient responsible for the resting potential and action and uptake and release of neurotransmitters, and as a consequence, a decrease of Na^+,K^+-ATPase activity critically affects neurotransmitter signaling, neural activity, as well as animal behavior (Moseley et al., 2007). For instance, the Na^+,K^+-ATPase inhibitor ouabain elicits electrographically-recorded seizures in mice (Doggett et al., 1970), and Na^+,K^+-ATPase activity and phosphorylation state of serine 943 (Ser^{943}) at the α subunit strongly correlate with the latency to seizures induced by pentylenetetrazol in mice (Marquezan et al., 2013). Moreover, mutations in the Na^+,K^+-ATPase α subunit gene have been found in mice and humans presenting epileptic phenotype (Clapcote et al., 2009; Deprez et al., 2008; Poulsen et al., 2010). Nevertheless, there is little evidence on the mechanisms underlying a decrease in Na^+,K^+-ATPase activity after SE. Therefore, the present study aimed to test the hypothesis that a decrease in Na^+,K^+-ATPase after SE is associated with changes in the alpha subunit phosphorylation and/or redox state.

Methods

Adult male C57BL/6 male mice (12—20 g) with 30—45-day-old were used All animal experimentation reported in this study has been conducted in accordance with national and international legislation and with the approval of the Institutional Committee on Animal Use and Care of the Federal University of Santa Maria (process #37/2012).

SE was induced by pilocarpine following standard procedures (Borges et al., 2003) and neurochemical analyzes were performed at two months after SE. For this purpose animals were euthanized by pentobarbital (180 mg/kg, i.p.) and had their brain exposed by the removal of the parietal bone. The hippocampus was rapidly dissected on an inverted ice-cold Petri dish and homogenized in the proper solution for each subsequent neurochemical analyzes. Given the small size of mouse hippocampus and therefore the limited amount of sample per animal we used different subjects in each set of neurochemical analysis. Detail of the animals and reagents used, procedures for SE, Na^+,K^+-ATPase activity measurements, immunodetection of Na^+-K^+-ATPase α subunit, immunoprecipitation and statistical analyzes are described in the Supplemental methods section.

Results

Statistical analyzes revealed a decrease of total Na^+,K^+-ATPase activity in the mice hippocampus two months after pilocarpine-induced SE [$t(6) = 2.979$; $P < 0.05$—Fig. 1A]. In addition, we investigated whether some α isoform is selectively decreased following SE. We found that $\alpha1$ [$t(6) = 3.240$; $P < 0.05$—Fig. 1B] and $\alpha2/\alpha3$ [$t(6) = 2.546$; $P < 0.05$—Fig. 1C] Na^+,K^+-ATPase activities are decreased in the hippocampus two months after SE. No changes in Na^+,K^+-ATPase activities were found at the other time points analyzed (24 h or 7 days after SE).

Functional analysis of the ATP dependence of Na^+,K^+-ATPase activity revealed that the K_m for ATP of total or $\alpha1$ Na^+,K^+-ATPase activities was not altered following pilocarpine-induced SE (Fig. 2A and B, respectively). On the other hand, a significant increase in the K_m for ATP of the $\alpha2/\alpha3$ isoforms was found two months after SE [$t(8) = 2.722$; $P < 0.05$—Fig. 2C].

In order to investigate the mechanism underlying decreased Na^+,K^+-ATPase activity after pilocarpine-induced SE we measured Na^+,K^+-ATPase α subunit immunoreactivity and its state of phosphorylation at Ser^{943}. Statistical analyzes revealed that α subunit immunoreactivity was not altered in hippocampus of epileptic mice (Fig. 3A), suggesting that the currently reported decrease of Na^+,K^+-ATPase activity was not due to a reduction in enzyme content. In addition, phosphorylated Ser^{943} immunoreactivity did not change after SE (Fig. 3B and C).

Since decreased expression or altered Ser^{943} phosphorylation of Na^+,K^+-ATPase α subunit seems not to underlie the decrease in enzyme activity, we investigated whether oxidative modifications of the α subunit were associated with decreased Na^+,K^+-ATPase activity two months after SE. Na^+,K^+-ATPase α subunit was immunoprecipitated from mice hippocampus and the oxidative stress markers 3-NT, HNE and protein carbonyls were measured. Interestingly, we found an increase in the nitration ratio of Na^+,K^+-ATPase α subunit [$t(8) = 3.016$; $P > 0.05$—Fig. 4A]. On the other hand, no