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SHORT COMMUNICATION

Pentylenetetrazol-induced seizures are associated with Na^+,K^+-ATPase activity decrease and alpha subunit phosphorylation state in the mice cerebral cortex

Bárbara P. Marquezan[a], Vinícius R. Funck[a], Clarissa V. Oliveira[a], Letícia M. Pereira[a], Stífani M. Araújo[b], Micheli S. Zarzecki[b], Luiz Fernando F. Royes[a,c], Ana Flávia Furian[a,d], Mauro S. Oliveira[a,b,*]

[a] *Federal University of Santa Maria, Pharmacology Graduate Program, Department of Physiology and Pharmacology, Santa Maria 97105-900, Brazil*
[b] *Federal University of Pampa at Itaqui, Itaqui 97650-000, Brazil*
[c] *Federal University of Santa Maria, Department of Sports Methods and Techniques, Santa Maria 97105-900, Brazil*
[d] *Federal University of Santa Maria, Department of Food Technology and Science, Santa Maria 97105-900, Brazil*

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Summary The present study aimed to investigate whether Na^+,K^+-ATPase activity and phosphorylation state of the catalytic α subunit are altered by pentylenetetrazol (PTZ)-induced seizures. PTZ (30, 45 or 60 g/kg, i.p.) was administered to adult male Swiss mice, and Na^+,K^+-ATPase activity and phosphorylation state were measured in the cerebral cortex 15 min after PTZ administration. Na^+,K^+-ATPase activity significantly decreased after PTZ-induced seizures (60 mg/kg). Immunoreactivity of phosphorylated Ser943 at α subunit was increased after PTZ-induced seizures. A significant positive correlation between Na^+,K^+-ATPase activity and latency to myoclonic jerks and generalized seizures was found. Conversely, a strong negative correlation between Ser943 phosphorylation and latency to generalized seizures was detected. Given the role of Na^+,K^+-ATPase as a major regulator of brain excitability, Ser943 at Na^+,K^+-ATPase α subunit may represent a potentially valuable new target for drug development for seizure disorders.

* Corresponding author at: Federal University of Santa Maria, Department of Physiology and Pharmacology, Pharmacology Graduate Program, Av. Roraima, 1000, Santa Maria 97105-900, Brazil. Tel.: +55 55 3220 9378.
E-mail address: ms.oliveira@ufsm.br (M.S. Oliveira).

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Introduction

Na^+,K^+-ATPase (EC 3.6.3.9) is a plasma membrane protein which is expressed in virtually all living cells (Skou and Esmann, 1992). By driving sodium export and potassium import across the plasma membrane, Na^+,K^+-ATPase plays a key role in the maintenance and regulation of electrolyte homeostasis in both intracellular and extracellular environments (Skou and Esmann, 1992).

In the mammalian central nervous system, Na^+,K^+-ATPase activity significantly accounts for the maintenance of the electrochemical gradient across the plasma membrane underlying resting and action potentials as well as neurotransmitter release and uptake (Moseley et al., 2007). Accordingly, a decrease of Na^+,K^+-ATPase activity critically affects neurotransmitter signaling, neural activity, as well as animal behavior (Moseley et al., 2007). In addition, it has been suggested that Na^+,K^+-ATPase plays a role in several neurological disorders, including seizure activity and epilepsy (Aperia, 2007; Benarroch, 2011). In this context, ouabain elicits electrographically-recorded seizures in rats (Bignami and Palladini, 1966) and mutations in the Na^+,K^+-ATPase α subunit gene have been associated with epilepsy in mice (Clapcote et al., 2009) and humans (Deprez et al., 2008; Poulsen et al., 2010a). These studies are, to some degree, in agreement with those that have shown that prostaglandin E_2 decreases Na^+,K^+-ATPase activity (Oliveira et al., 2009), increases phosphorylated Na^+,K^+-ATPase α subunit Ser943 immunoreactivity (Oliveira et al., 2009) and facilitates PTZ-induced seizures (Oliveira et al., 2008). However, it is not clear whether alterations in the phosphorylation state of Ser943 are associated to seizures, in an individual basis. Therefore, considering that protein phosphorylation is an important mechanism of Na^+,K^+-ATPase activity control (Poulsen et al., 2010b, 2012), and the emerging role of Na^+,K^+-ATPase as a putative therapeutic target for selected neurological disorders (Aperia, 2007; Benarroch, 2011), the present study aimed to investigate whether the phosphorylation state of the catalytic α subunit is altered by pentylenetetrazol (PTZ), a classical convulsant agent that has been successfully used in the study of seizure mechanisms and screening of anticonvulsant drugs.

Methods

Adult male Swiss mice 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 #51/2010). Detail of the animals and reagents used, seizure evaluation, Na^+,K^+-ATPase activity measurements, immunodetection of Na^+,K^+-ATPase α subunit and statistical analyses are described in the Supplemental Methods section.

Seizure evaluation

Animals were injected intraperitoneally with PTZ (30, 45 or 60 mg/kg) or its vehicle (0.9% NaCl), and were monitored for 15 min for the occurrence of behavioral seizures. During the 15 min observation period, the latency to myoclonic jerks and generalized tonic-clonic seizures was recorded.

Neurochemical analyses

Immediately after seizure evaluation (15 min after PTZ administration) the animals were killed by decapitation and the cerebral cortex was rapidly dissected on an inverted Petri dish placed on ice, and gently homogenized in an appropriated solution for determination of Na^+,K^+-ATPase activity or immunodetection of Na^+,K^+-ATPase α subunit as described in detail by Oliveira et al. (2009).

Results

Administration of PTZ (45 and 60 mg/kg) caused the appearance of myoclonic jerks (Fig. 1A), but only 60 mg/kg PTZ elicited generalized tonic-clonic seizures (Fig. 1B). The administration of PTZ (60 mg/kg) decreased Na^+,K^+-ATPase activity by 37.65% in the cerebral cortex [$F(3,40) = 2.932$; $P < 0.05$; Fig. 1C]. Interestingly, Pearson correlation analyses revealed a strong positive correlation between Na^+,K^+-ATPase activity in the cerebral cortex and the latency to PTZ-induced myoclonic jerks ($r = 0.7425$; $P < 0.01$ — Fig. 1D) or generalized tonic-clonic ($r = 0.7799$; $P < 0.005$ — Fig. 1E) seizures.

In order to investigate whether the PTZ-induced decrease of Na^+,K^+-ATPase activity was due to a decrease in the levels of available enzyme molecules, we measured the total Na^+,K^+-ATPase α subunit immunoreactivity in the samples. Statistical analysis revealed that PTZ did not alter total α subunit immunoreactivity [$t(7) = 0.1715$; $P > 0.05$] (Fig. 2A), indicating that the content of the catalytic α subunit is similar between PTZ-treated and control animals.

Since Na^+,K^+-ATPase activity is regulated by phosphorylation of the α subunit at Ser943 (Poulsen et al., 2010b), we investigated whether PTZ-induced seizures are accompanied by changes in the phosphorylation state of this residue. PTZ administration significantly increased Ser943 phosphorylation by 82.5% [$t(10) = 2.616$; $P < 0.05$] (Fig. 2B). Pearson correlation analysis revealed a strong negative correlation between Ser943 phosphorylation and latency to generalized tonic-clonic seizures induced by PTZ (Pearson $r = -0.88$; $P < 0.005$ — Fig. 2C) in an individual basis.

Discussion

Na^+,K^+-ATPase is a major regulator of brain excitability. Accordingly, current evidence supports that decreased Na^+,K^+-ATPase enhances neuronal excitability and facilitate convulsions. In fact, ouabain increases the frequency of spontaneous postsynaptic potentials and decreases the amplitude and duration of CA3 pyramidal cell afterhyperpolarizations in hippocampal slices (Haglund and Schwartzkroin, 1990), and blocks an outward current which is critical to neuron hyperpolarization between bursts of action potentials (Johnson et al., 1992). Moreover, ouabain induces electrographically-recorded seizures in rats (Bignami and Palladini, 1966), and a point mutation in the Na^+,K^+-ATPase $\alpha 3$ isoform results in a phenotype which is

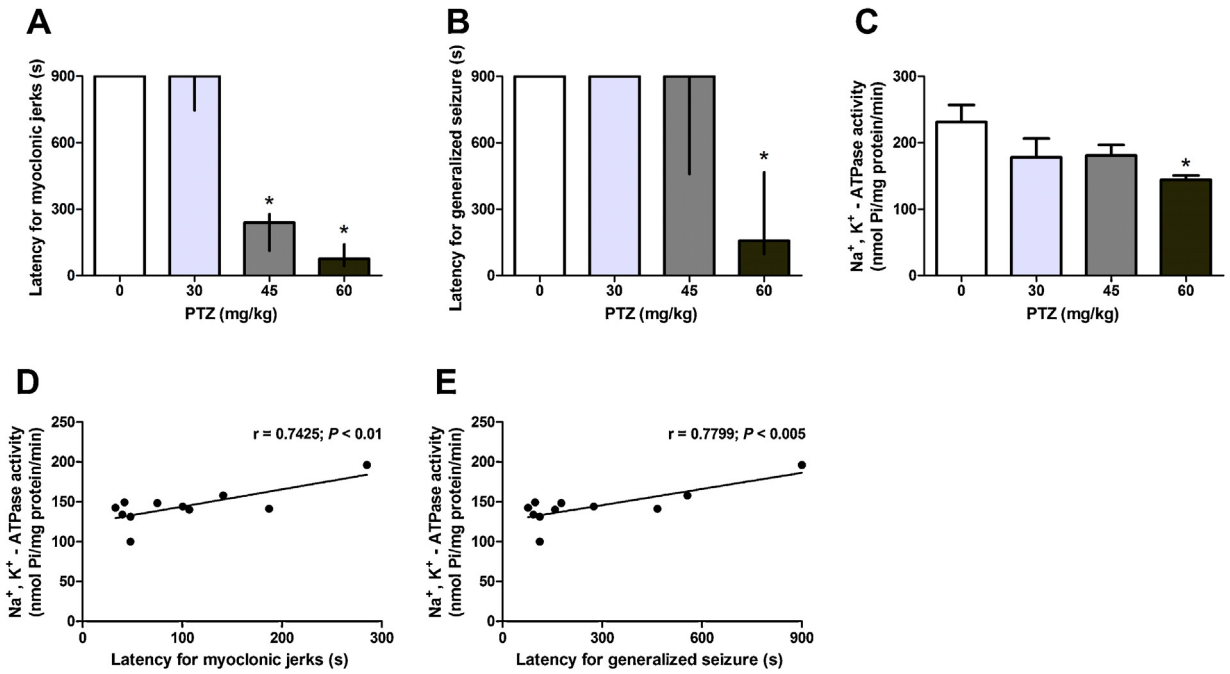

Figure 1 Latency to myoclonic jerks (A) and generalized seizures (B) induced by increasing doses of PTZ and effect of PTZ on Na^+,K^+-ATPase activity (C). Correlation analyses between Na^+,K^+-ATPase activity and the latency to PTZ-induced myoclonic jerks and generalized seizures are depicted in panels D and E, respectively. Behavioral data are presented as median and interquartile ranges and Na^+,K^+-ATPase activity data are presented as mean + SEM for $n = 11$ in each group, from four different experiments. *Indicates a significant difference compared with control group.

characterized by both focal and generalized seizures in mice (Clapcote et al., 2009). It is also remarkable that decreased Na^+,K^+-ATPase activity has been found in the post-mortem epileptic human brain (Grisar et al., 1992), and that seizures are a hallmark of the phenotype associated with mutations in the Na^+,K^+-ATPase α subunit gene in humans (Deprez et al., 2008; Poulsen et al., 2010a). Altogether, these findings constitute compelling evidence that direct Na^+,K^+-ATPase activity inhibition causes brain hyperexcitability *in vitro* and elicit seizures *in vivo*.

Protein phosphorylation appears to be one an important mechanism for the short-term regulation of Na^+,K^+-ATPase (Poulsen et al., 2010b). Several phosphorylation sites have been identified in the catalytic α subunit of Na^+,K^+-ATPase, and most of them have been implicated in the regulation of enzyme activity in response to hormones and neurotransmitters in a cell- and isoform-specific manner. For instance, Ser943 in the α subunit of Na^+,K^+-ATPase is a well-characterized phosphorylation site for protein kinase A (PKA) (Cheng et al., 1997a; Poulsen et al., 2012).

Although the physiological aspects of Na^+,K^+-ATPase phosphorylation by different protein kinases have been systematically investigated (Poulsen et al., 2010b), the role for changes in the Na^+,K^+-ATPase phosphorylation state in pathological conditions has been much less studied, particularly in what regards the brain Na^+,K^+-ATPase. In fact,

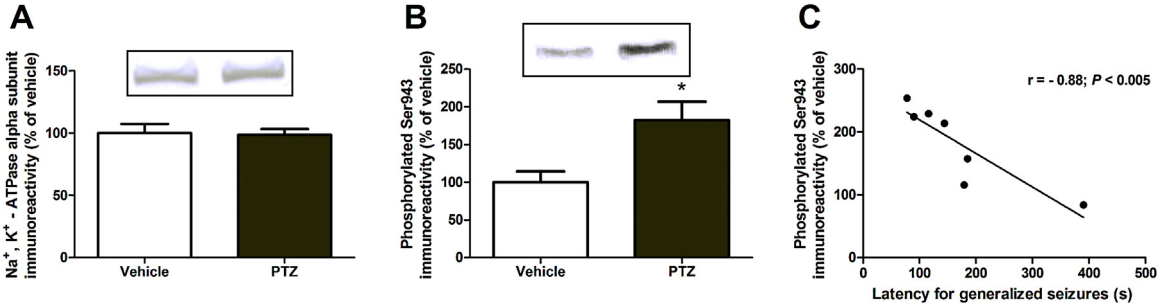

Figure 2 Effect of PTZ (60 mg/kg) on total Na^+,K^+-ATPase α subunit immunoreactivity (A), immunocontent of Na^+,K^+-ATPase α subunit phosphorylated at Ser943 (B), and correlation analysis between the immunocontent of Na^+,K^+-ATPase α subunit phosphorylated at Ser943 and the latency to PTZ-induced generalized seizures (C). Data are mean + SEM for $n = 4$—5 in each group for total Na^+,K^+-ATPase α subunit immunoreactivity and $n = 5$—7 for immunocontent of Na^+,K^+-ATPase α subunit phosphorylated at Ser943, from four different experiments. *Indicates a significant difference compared with control group.

to the best of our knowledge, the present study constitutes the first report on the increase in phosphorylation of the catalytic α subunit of Na^+,K^+-ATPase at Ser943 following seizures. To some extent, our present results are in agreement with those by Yang et al. (2007), who have shown that hypoxic-ischemic neuronal injury in the striatum of newborn piglets is accompanied by Na^+,K^+-ATPase activity decrease and concomitant increase in phosphorylation of Ser943.

In this context, functional studies on the effects of PKA-mediated phosphorylation of Na^+,K^+-ATPase have demonstrated that phosphorylation of Ser943 is often linked with decreased enzyme activity. In fact, the PKA activator forskolin increases Ser943 phosphorylation and concomitantly reduces Na^+,K^+-ATPase activity, as measured by a decrease in ATP hydrolysis and $^{86}Rb^+$ transport (Cheng et al., 1997b). Similar results have been found with the use of a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (Cheng et al., 1997a). Interestingly, the level of α subunit phosphorylation at Ser943 is associated with a decrease in Na^+,K^+-ATPase activity which inversely correlates with the magnitude of phosphorylation (Cheng et al., 1997a), further indicating that phosphorylation of Na^+,K^+-ATPase at Ser943 is associated with a decrease in enzyme activity. Regarding this point, and in light of the presently reported strong negative correlation between Ser943 phosphorylation and the latency to PTZ-induced generalized seizures, it is possible that phosphorylation of Ser943 at the α subunit of Na^+,K^+-ATPase represents a critical mechanism underlying the decrease in Na^+,K^+-ATPase activity which accompanies PTZ-induced seizures. In this context, it is worth mentioning that prostaglandin E_2, which facilitate PTZ-induced seizures (Oliveira et al., 2008), decreases Na^+,K^+-ATPase activity and increases Ser943 phosphorylation (Oliveira et al., 2009).

It is also important to reflect on the role played by the different Na^+,K^+-ATPase α isoforms in PTZ-induced seizures. Three isoforms of the Na^+,K^+-ATPase α subunit have been detected in the brain; while the α1 isoform is found in many cell types, the α2 isoform is predominantly expressed in glia and hippocampal pyramidal cells and the α3 isoform is expressed only in neurons (Moseley et al., 2007). Considering that the antibodies used here are not able to discriminate between the different Na^+,K^+-ATPase α subunits, at the present it is not possible to conclude if a particular Na^+,K^+-ATPase α isoform is being phosphorylated at Ser943 after PTZ administration. Moreover, it is not possible to anticipate whether only neuronal Na^+,K^+-ATPase plays a role in PTZ-induced seizures, since it has been demonstrated that astrocytes may contribute to initiation of seizure activity even when synaptic activity is blocked (Tian et al., 2005), and therefore it is possible that decreased Na^+,K^+-ATPase activity in both neurons and astrocytes plays a role in PTZ-induced seizures.

In summary, in the present study we showed that Na^+,K^+-ATPase activity in the mice cerebral cortex significantly decreases following PTZ-induced seizures, regardless of changes in the content of the catalytic α subunit of the enzyme. In addition, phosphorylation of Ser943 at α subunit significantly increased after the seizures induced by PTZ. Interestingly, Na^+,K^+-ATPase activity correlated positively with the latency to clonic and generalized tonic-clonic seizures, whereas Ser943 phosphorylation levels and the latency to PTZ-induced generalized tonic-clonic seizures correlated negatively. Given the role of Na^+,K^+-ATPase as a major regulator of brain excitability, Ser943 at Na^+,K^+-ATPase α subunit may represent a potentially valuable new target for drug development for seizure disorders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eplepsyres.2013.03.007.

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