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THE SYNERGY OF RAPAMYCIN AND PIOGLITAZONE ANTISEIZURE ACTION IN PENTYLENETETRAZOL (PTZ)-KINDLED RATS

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Abstract

The investigation was performed on 28 male Wistar rats. Through three weeks of pentylenetetrazol (35.0 mg/kg, i.p.) administrations model of kindled generalized tonic-clonic seizures was created.

It was established that combined treatment of fully kindled rats during ten days with rapamycin (1.0 mg/kg, i.p.) and pioglitazone (50.0 mg/kg, i.p.) prevented generalized seizure fits in 7 out of 8 animals($z=2.870$, $P=0.004$). Simultaneously the prevention of ictal discharges generation in the frontal cortex and ventral hippocampus was registered. Seizure severity was reduced by two times when compared with the control group ($P<0.05$) and significantly less by 30.6% when compared with the one registered in rats treated with rapamycin (2.0 mg/kg, i.p.).

The conclusion was made that the antiseizure effects of pioglitazone and rapamycin are intensified under conditions of their combined administration.

Keywords: *kindling, epileptic activity, pentylenetetrazol, rapamycin, pioglitazone.*

Introduction

Inhibitors of the mammalian target of rapamycin (mTOR) pathway are recognized as perspective antiepileptic compounds [1-4]. Antiseizure action of rapamycin – a well-known mTOR inhibitor, has been shown on kainic acid-induced continuous seizures [5], epileptic status induced with pilocarpine [6, 7] as well as on status epilepticus induced with brain structures electrical stimulations [8]. Vice versa, activation of the mTOR pathway intensified brain epileptization in genetic and acquired rodent models of epilepsy [5, 9]. Antiseizure effectiveness of pioglitazone was established on PTZ-induced kindling [10], scopolamine-induced [11], and febrile-seizures [12].

Both compounds – rapamycin and pioglitazone, as an agonist of Peroxisome Proliferator-Activated Receptors –γ (PPARγ), are different from classical antiepileptic drugs and cause a broad spectrum of effects, including modulation of cell growth, proliferation, autophagy, and epigenetic effects [13-15].

Considering numerous effects of modulation of nervous tissue with mTOR blockers and agonists of PPARγ, it was reasonable to investigate the effectiveness of combined usage of rapamycin and pioglitazone upon the model of chronic epileptic activity. Earlier, we have shown that such an approach resulted in the synergy of rapamycin and axitinib (inhibitor of tyrosine kinase B) establishment on the model of PTZ kindled seizures [16].

Hence, the aim of our work was to investigate the effects of rapamycin upon pentylenetetrazol (PTZ)-induced kindled seizures.

Methods

Experimental animals

Experiments were performed on 28 male Wistar rats with an initial body weight of 180-250 g. Animals were kept in standard conditions (constant temperature 230 C, relative humidity 60%, 12 h dark/light cycles, standard diet, and tap water were given ad libitum) and were acclimatized to laboratory conditions at least seven days before the experiment. All experiments were carried out following the National Institute of Health Guidelines for the care and use of laboratory animals and the European Council Directive on 24 November 1986 for Care and Use of Laboratory Animals (86/609/EEC). The experiments were approved by Danylo Halytsky Lviv National Medical University (approval No. 3 dated 14/03/2016) before the study.

Epilepsy model

Kindled seizures were induced, as described previously [17]. PTZ ("Sigma Aldrich") was given intraperitoneally (i.p.) daily at a dose of 35.0 mg/kg for 21 days. The severity of seizures was evaluated according to the following criteria: 0, absence of symptoms of seizures; 1, facial tremor and separate myoclonic jerks; 2, whole-body clonic seizures; 3, clonic seizures of the whole body with rearings; 4, generalized clonic-tonic seizures with rearings and falling; and 5, repeated seizures as at stage 4 or lethal outcome as a result of a seizure fit. As a response to both 20th and 21t PTZ injections, those rats, which failed into generalized fits, were taken for the inclusion of an animal to further observations.

Groups of animals

Seven rats, which were PTZ-administered during three weeks (35.0 mg/kg, i.p.) with the consequent DMSO i.p. 10 days treatment, were used as a control (Fig. 1). Experimental groups of fully kindled rats were treated during ten days with rapamycin (2.0 mg/kg, i.p.), pioglitazone (100.0 mg/kg, i.p.) and combined rapamycin (1.0 mg/kg, i.p.) and pioglitazone (50.0 mg/kg, i.p.) (total – 21 rats).

Surgery

In animals, anesthesia was performed with i.p. ketamine administration (100 mg/kg, i.p., Farmak, Ukraine). Rats were placed in a stereotactic apparatus, "SEZh-5" (Kyiv, Ukraine). A 0.5% Novocain solution infiltration (Darnitsa, Ukraine) was used for local anesthesia before incision. The head was shaved and cleaned with iodine before incision [16, 17]. EEG acquisition and stimulation electrodes were placed in relation to the bregma after skin dissection (2 cm along a middle sagittal

axis) and the removal of all soft tissues from the skull surface. A 1.5-2.0 mm burr hole for EEG acquisition and a 3.0-4.0 mm hole for stimulation electrodes were drilled through the cranium with a standard dental portable drill (Colt 1, Charkov, Ukraine).

The nichrome monopolar electrodes were implanted in the ventral hippocampus (AP=-4.3; ML=4.5; DV=-8.0), and frontal cortex (AP=1.7; ML=2.0; DV=-1.0) of both hemispheres, according to the rat brain atlas [18]. The reference electrode was located in the nasal bones. The electrodes were fixed on the cranial surface with dental cement. To prevent dehydration, 5.0 ml of 0.9% NaCl solution heated to 35°C was injected i.p. at the end of surgery. Penicillin potassium salt (100,000 IU/kg, intramuscular injection) was administered every 12 h during 48 h post-surgery to prevent infection. Animals were allowed to recover for 10 to 14 days after surgery before observations.

EEG data acquisition and analysis

The analog data were acquired using computer electroencephalograph DX-5000 (Charkov, Ukraine), and the data were digitized at a 256 Hz sampling rate. The time constant was 0.1, and the low-pass filter was set at 70 Hz. Extremely low frequency and high amplitude (excessive) waves on EEG synchronously appearing in all leads coinciding with behavioral movements were treated as artifacts and excluded from the analysis. The polygraph recordings were analyzed offline visually, and epochs containing artifacts were eliminated.

Visual control of electrodes location

All experimental animals were euthanized with Nembutal (100.0 mg/kg, i.p.). Upon completion of the experiment, the visual quality control of the electrode placement was *extempore* on the gently removed tissue (no transcardial perfusion). For this purpose, electrocoagulation was performed in the electrode placement area, applying the direct current with an amplitude of 5.0 mA over 30 s and using the electrodes as an anode [17].

Investigated compounds administration

In accordance with the study design (Fig. 1), pioglitazone (Lilly S.A., Spain) was administered in doses of 100.0 mg/kg, i.p. and 50.0 mg/, i.p. Rapamycin (Pfizer, USA) was administered in doses of 2.0 and 1.0 mg/kg, i.p. Both compounds were dissolved in DMSO and delivered in 0.20-0.30 ml in 60 min before PTZ administration during ten days. Lower doses were used when a combined version of treatment was used (Fig. 1).

Statistics

Values were compared using one-way ANOVA and Newman-Keuls test for the latency of seizures; Kruskal-Wallis followed with a post hoc test was used for seizure severity; and "z" criteria for comparing two proportions. Results were presented as M±S.E.M. The Shapiro-Wilk test for normality was used for the latent period. *P* values <0.05 were considered significant. Only observations falling between median ± 3.0 SD of the sample were included in the dataset to avoid outliers' influence.

Results

Repeated PTZ (35.0 mg/kg, i.p.) administrations caused the appearance of the first seizures after the 2d-5th injection. The intensity of seizure manifestations raised in further epileptogenic injections and generalized tonic-clonic seizure fits with fallings and postictal animals depression appeared in 12-19th administration. The latency of seizures was 65.7±3.85 sec, and their severity – 4.7±0.2 scored points in fully kindled rats (Fig.2).

In rapamycin (2.0 mg/kg, i.p.) treated rats, the latency of first seizures exceeded such one in the control group by 24.5% (P>0.05) (Fig. 2, A). The latent period of seizures in rats with pioglitazone administration (100.0 mg/kg, i.p.) prevailed control data by 30.5% (P>0.05), while in rats which both drugs were administered in reduced doses (1.0 mg/kg, i.p. and 50.0 mg/kg, i.p. for rapamycin and pioglitazone correspondently) the difference was 42.7% (P<0.05) (Fig.2, A).

In rapamycin-treated rats (2.0 mg/kg, i.p.), generalized seizure fits were registered in 5 out of 7 rats, and seizure severity and average severity of seizures was 27.2% less when compared with the control group (P<0.05) (Fig.2, B). In rats treated

with pioglitazone (100.0 mg/kg, i.p.), generalized seizure fits were registered in 2 out of 6 animals (z=2.004, P=0.045), and average seizure severity was reduced by 32.7% in comparison with the control group (P<0.05). Combined treatment with rapamycin (1.0 mg/kg, i.p.) and pioglitazone (50.0 mg/kg, i.p.) prevented generalized seizure fits in 7 out of 8 animals (z=2.870, P=0.004), and seizure severity was reduced by two times when compared with the control group (P<0.05) (Fig. 2, B). At the same time, seizure severity was significantly less by 30.6% when compared with the one registered in rats treated with rapamycin (2.0 mg/kg, i.p.) (Fig.2, B).

The duration of ictal potential, which was registered in the frontal cortex and ventral hippocampus in the control group, was 25.37±3.12 sec (Fig. 3, A). Duration of ictal discharge was shortened up to 11.7±2.14 sec under conditions of rapamycin (2.0 mg/kg, i.p.) treatment (P<0.05) (Fig.3, B). In 4 out of 6 rats treated with pioglitazone (100.0 mg/kg, i.p.), spike-wave discharges grouped in trains were registered during fit-like rhythmic clonic seizures (Fig.3, C). The average duration of such trains was 15.3±4.2 sec (P<0.05). Ictal discharges were prevented in 7 out of 8 rats under conditions of combined rapamycin (1.0 mg/kg, i.p.) and pioglitazone (50.0 mg/kg, i.p.) treatment (z=2.870, P=0.004), and spike discharges generated with the frequency of 10-20 per min and with magnitude from 350 up to 800 mcV were registered (Fig.3, D).

Discussion

Hence, gained data showed that administration of rapamycin and pioglitazone prevented PTZ-induced kindled seizures in rats. Namely, each of the drugs delivered during ten days blocked generalized clonic-tonic fits. Also, ictal discharges registered in the frontal cortex and ventral hippocampus were shortened or abolished – the fact which favors the central character of antiseizure action of both drugs. Complete prevention of ictal discharges along with the protection from seizure fits and a two-fold reduction of seizure severity was registered when both drugs were administered in two times fewer dosages than in the case of their separate administration. Hence, obtained data point on strengthening antiseizure action of rapamycin and pioglitazone when used in a combined fashion.

Considering possible mechanisms of the facilitation of antiseizure action of drugs, the next should note. Thus, mTOR blockade caused antioxidant effects [19, 20]. Earlier, we have reported antioxidant effects of rapamycin in PTZ – kindled rats along with the antiseizure effects precipitation [16]. Pioglitazone also reduced oxidative stress [21-23] and caused antiseizure effects on PTZ-induced kindled seizures [10], scopolamine-induced [11], and febrile seizures [12]. Even more, synergy with regard to the antioxidative action of rapamycin and PPAR gamma activation was reported [24].

Interestingly that pioglitazone suppressed mTOR–depended on mechanisms and prevented the increase of IL-1 β and IL-6 on the model of PTZ-induced status epilepticus in rats [25]. Hence, mechanisms of strengthening antiseizure effects might be realized via active elements of the common pathway/mode of action of rapamycin and pioglitazone.

The established strengthening of antiseizure effects of rapamycin under conditions of inhibition of tyrosine kinase B with axitinib favors the involvement of BDNF/Trk signaling pathway caused by both pioglitazone [21, 22] and rapamycin [26, 27].

Different types of protein kinases might be involved in the strengthening antiseizure effects of combined treatment with pioglitazone and rapamycin [28-30]. Protein kinase activity modulation underlies numerous widespread effects, including changes in neuropeptides level, which are known as mediators of brain antiepileptic system activity [31-35]. Such ones as endogenous ligands to opiate receptors and kyotorphin, which are potent analgetic and antiepileptic substances [36-39], should be mentioned as candidates for synergy-like mechanisms of the rapamycin and pioglitazone interaction.

Obtained data assume that combined administration of agonists of PPARγ and blockers of mTOR might have resulted in potentiated therapeutic effects concerning other forms of pathology, which are targets for treatment with such pharmacies.

Hence, rapamycin (2.0 mg/kg, i.p.) and pioglitazone (100.0 mg/kg, i.p.) administered during

ten days prevented generalized clonic-tonic seizures as well as ictogenesis in the frontal cortex and ventral hippocampus induced in PTZ-kindled rats. Ten days of treatment of kindled animals with two-fold reduced doses of rapamycin and pioglitazone resulted in strengthened preventive antiseizure effects.

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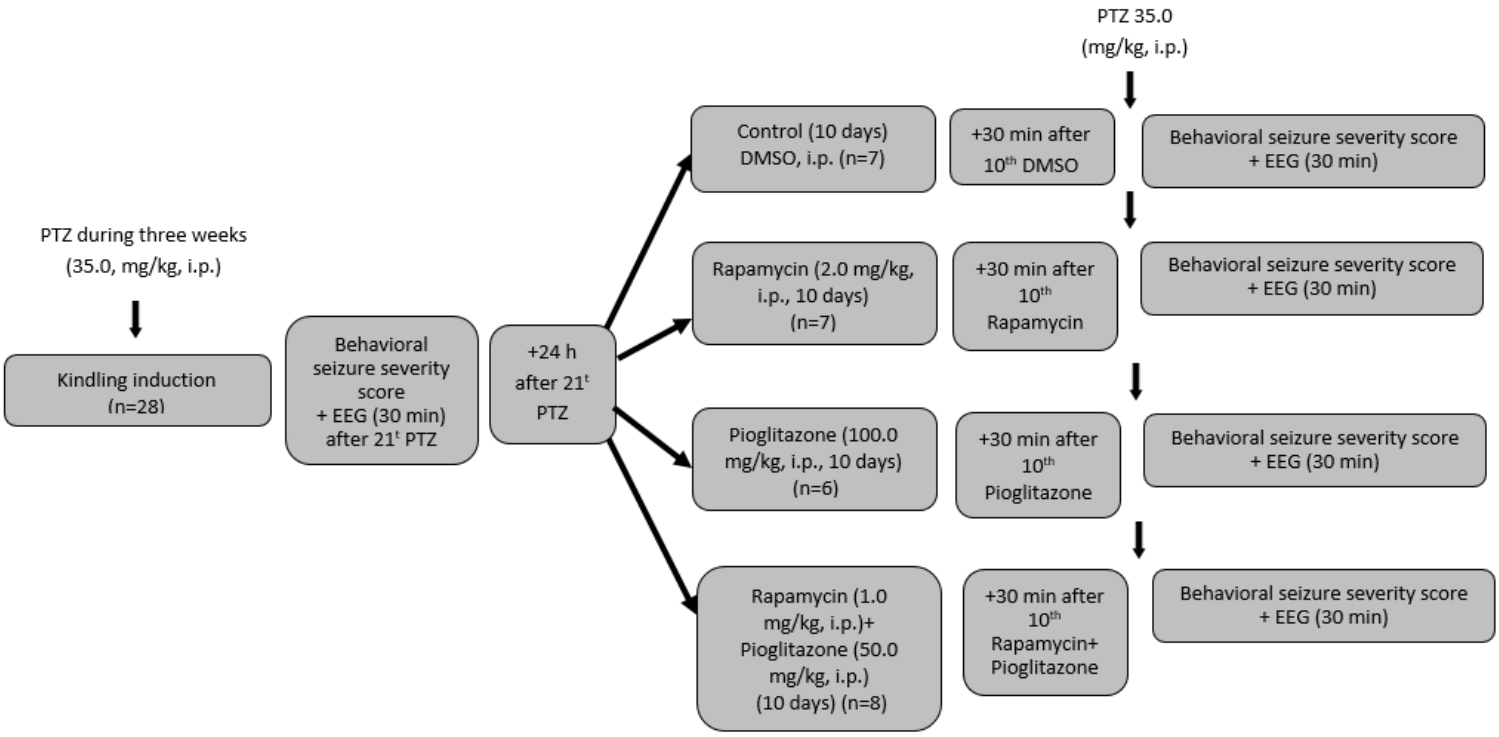
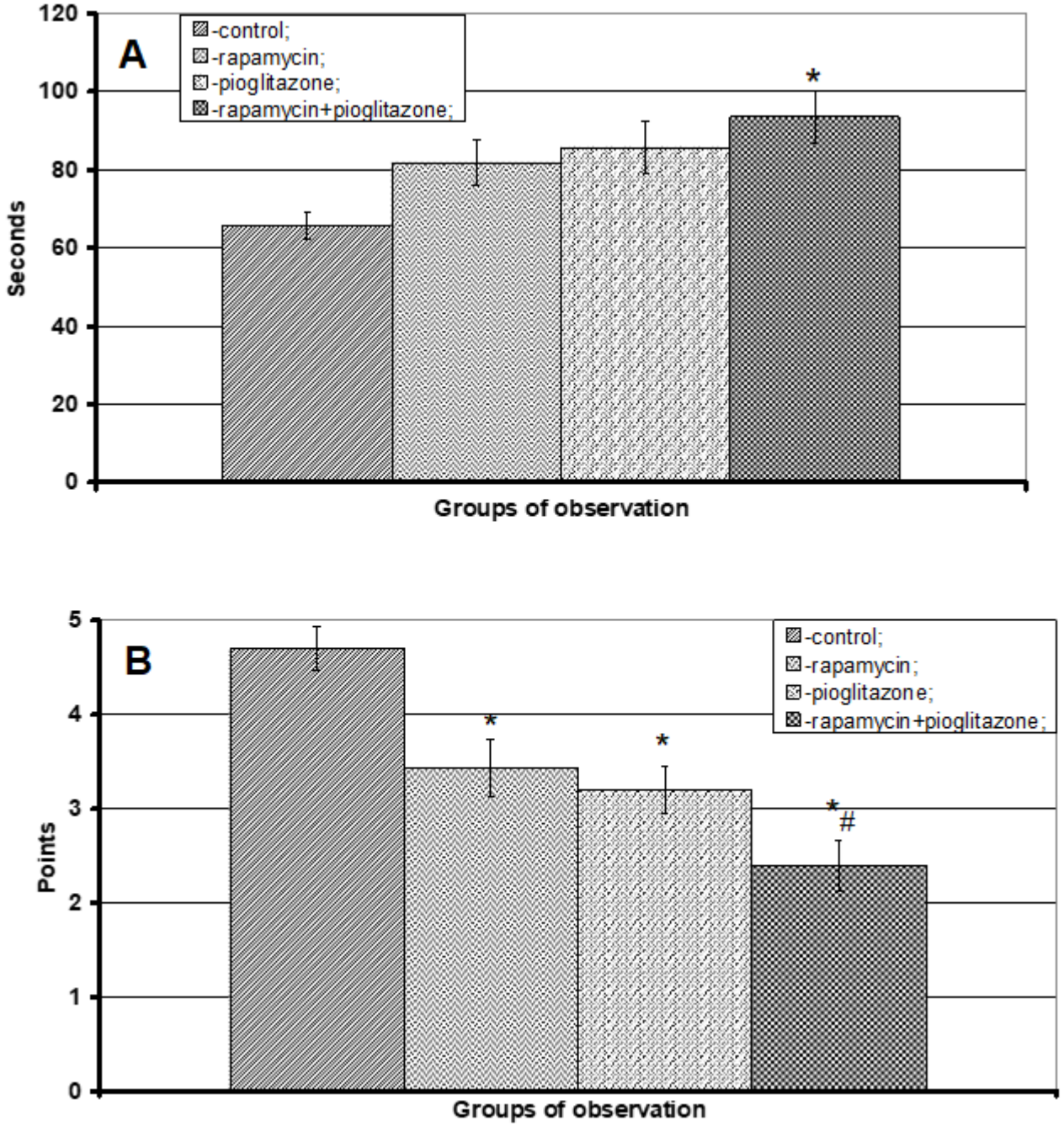
Figure 1.



Design of investigations.

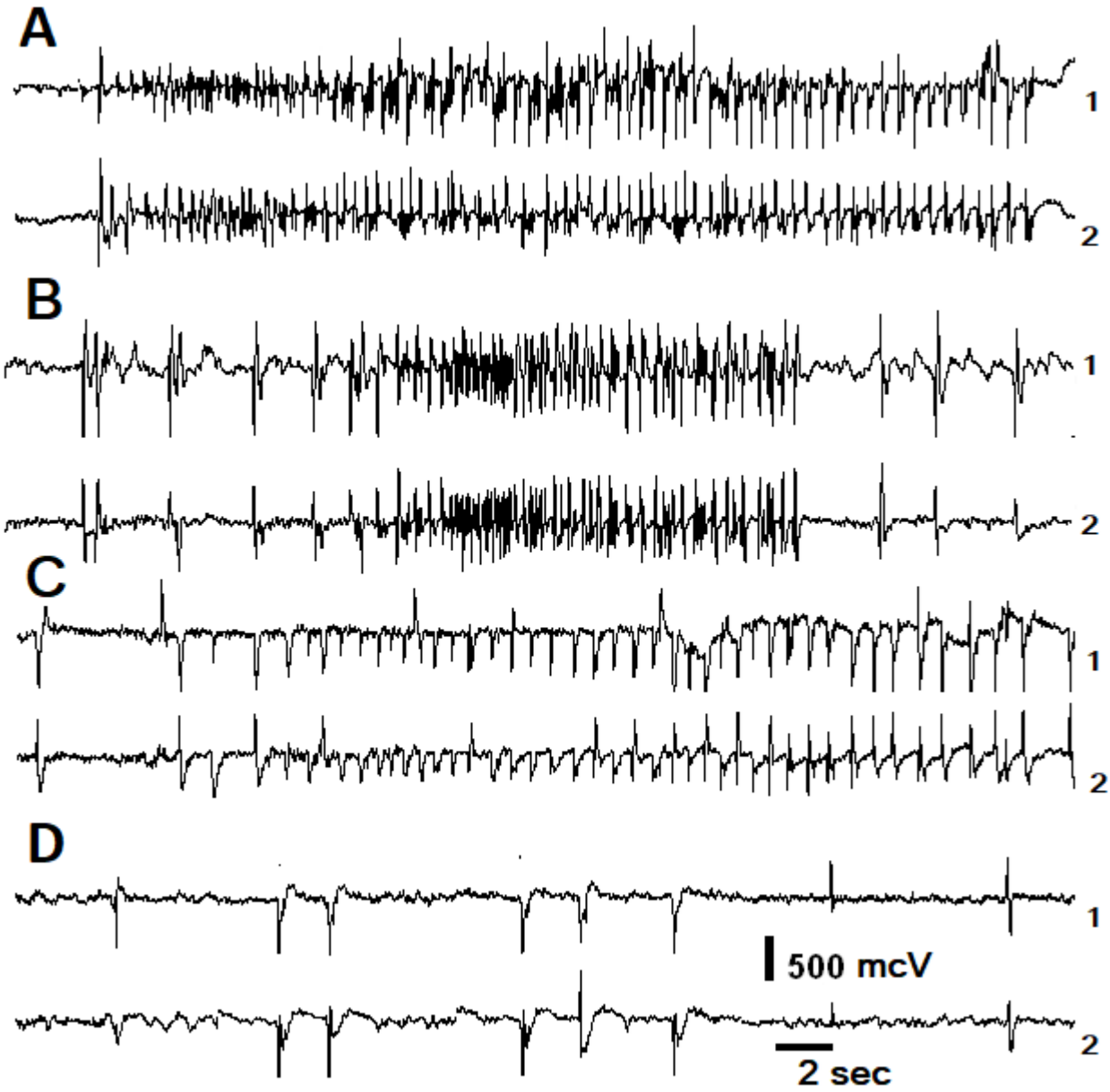
Figure 2.



The latency of first PTZ-kindled seizures (A) and seizure severity (B) under conditions of combined treatment with rapamycin and pioglitazone.

N o t e s: *- P<0.05 when compared with the control group, #-P<0.05 when compared with the group treated with rapamycin (ANOVA+ Newman-Keuls).

Figure 3.



PTZ-induced kindled electrographic manifestations in brain structures under conditions of treatment with Rapamycin and Pioglitazone.

N o t e s: 1- frontal cortex, and 2- ventral hippocampus of the left hemisphere.
A-control (25.0 min after PTZ); B-Rapamycin (2.0 mg/kg, i.p., 19.5 min after PTZ); C-Pioglitazone (100.0 mg/kg, i.p.), 22.5 min after PTZ; Rapamycin (1.0 mg/kg, i.p.) + Pioglitazone (1.0 mg/kg, i.p.), 27.0 min after PTZ.