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Original article

Influence of salbutamol on the anticonvulsant potency of the antiepileptic drugs in the maximal electroshock-induced seizures in mice

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ABSTRACT

Background: β_2-Adrenergic receptor agonists are widely used agents in the treatment of asthma or preterm labor. Since prevalence of asthma was shown to be higher in patients with epilepsy and modulation of noradrenergic system activity may modify epilepsy course, the aim of the present study was to examine the effect of salbutamol (SALB), one of the most commonly used β_2-adrenergic receptor agonist on the anticonvulsant potency of four classical antiepileptic drugs (AEDs): valproate (VPA), carbamazepine (CBZ), phenytoin (DPH) and phenobarbital (PB) in mice subjected to the maximal electroshock (MES)-induced seizures.

Methods: Seizures were caused by a current delivered through ear-clip electrodes. The influence of AEDs and SALB on animals' motor coordination and memory processes was also evaluated.

Results: Single SALB injection did not change, whereas 7 days SALB administration decreased seizure threshold in the MES-induced seizures in mice. Moreover, SALB injected *ip* for 1 day and for 7 days lowered the antiepileptic activity of PB in the MES-induced seizures in mice, but did not change the effect of other analyzed AEDs: VPA, CBZ or DPH. Butoxamine, a selective β_2-adrenergic receptor antagonist, reversed SALB influence on the activity of PB. SALB given alone or in combination with the tested AEDs did not affect animals' motor performance and memory after both single and 7 days administration.

Conclusions: Presented results show that SALB may decrease the antiepileptic efficacy of PB. A special caution is advised to patients with epilepsy receiving β_2-adrenergic receptors agonists in the pharmacotherapy of pulmonary and obstetrical disorders.

Introduction

Epilepsy is a chronic neurological disorder affecting around 0.5–0.7% of the population worldwide [1]. Reduction of seizure frequency and long remission time are the main goals in the therapy of epilepsy. Monotherapy is widely used and recommended in the pharmacotherapy of epilepsy since 70-ties and 80-ties of XXth century [2–4]. Reduction of drugs side effects [5], less pharmacokinetic drug interactions [1] and lower teratogenic potential [6] are the main clinical benefits of monotherapy. Despite these advantages monotherapy fails to control epilepsy in 30% of patients with focal epilepsy [7], whereas other studies report that more than 50% of patients do not respond to a single antiepileptic drug (AED) treatment [8]. Moreover, in some patients drug resistant epilepsy is diagnosed, when pharmacotherapy with two sequentially applied AEDs alone or in combination did not control disease symptoms [8]. This explains the need for safe and efficacious therapy in

Abbreviations: AEDs, antiepileptic drugs; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CBZ, carbamazepine; CS_{50}, median current strength; DPH, phenytoin; ED_{50}, median effective dose; GABA, γ-aminobutyric acid; MES, maximal electroshock; PB, phenobarbital; SALB, salbutamol; St 587, 2-(2-chloro-5-trifluoromethylphenylimino) imidazolidine; TD_{50}, median toxic dose; VPA, valproic acid.

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patients with epilepsy. Rational treatment should be determined according to accessible results from both, animal and clinical studies. Despite significant side effects conventional AEDs remain the basis of the therapy of epilepsy [9]. Since the number of available AEDs is still growing, the polytherapy of epilepsy is constantly evolving. Up-to-date, synergy was confirmed for many AEDs combinations, like phenytoin (DPH) and benzodiazepines [10–12], DPH and phenobarbital (PB) [13,14], DPH with PB and pregabalin [15], DPH and valproate (VPA) [16], VPA and carbamazepine (CBZ) [17] or ethosuximide [18]. Weaker antiepileptic efficacy was observed after combining VPA or CBZ with PB [17]. The evidence of using a combination of classic AEDs with novel antiepileptic agents is still discussed and depends on epilepsy model applied [19].

According to experimental data norepinephrine has modulatory effect in the seizures pathogenesis [20]. Lower norepinephrine levels and its receptors density were found in animals after seizure attacks [21,22]. Moreover, in the genetically prone epilepsy rats abnormalities in noradrenergic signaling were reported [23]. In cobalt–induced focal epilepsy model destruction of locus coeruleus in rats evoked increase in duration and intensity of seizure attacks [24]. The role of disturbances in noradrenergic neurotransmission was confirmed in the model of generalized [25], audiogenic [26] and kindled amygdala seizures [27]. Agents elevating norepinephrine level are claimed to have antiepileptic potential [28,29]. On the other hand, higher norepinephrine concentrations were shown to cause convulsions under special conditions, especially in the case of drug overdose [30].

Patients suffering from two or more disorders are in special danger of being exposed to drug interactions. In fact, Beghi recently reported that asthma occurs more frequently in patients with epilepsy [31]. What is interesting, *in utero* exposure to β_2-adrenergic receptors agonists during first or second trimester of pregnancy was shown to increase the risk of epilepsy in the future [32]. Among analyzed β_2-adrenergic receptors agonists the most commonly used agents were salbutamol and terbutaline [32].

The goal of this study was to analyze the effect of a single and 7-day administration of the β_2-adrenergic receptor agonist salbutamol (SALB) on the protective effect of four classical antiepileptic drugs (AEDs): carbamazepine (CBZ), valproate (VPA), phenobarbital (PB) and phenytoin (DPH) in the MES-induced seizures in mice. Moreover, experiments in mice with MES-induced seizures were carried out using butoxamine, a selective β_2-adrenergic antagonist. Additionally, the effect of SALB on AEDs' side effects was evaluated in the chimney test (to analyze animals' motor impairment) and passive avoidance test (to examine animals' long-term memory deficits).

Materials and methods

Animals

Tests were carried out on male Albino Swiss mice (T. Górzkowska, Warsaw, Poland), weighing 20–25 g at the time of the experiment. Animals were kept in standard laboratory conditions, at room temperature (21 °C ± 1 °C) and relative humidity of 55 ± 5%, with water and food (Murigran pellets, Bacutil, Motycz, Poland) available *ad libitum*. Experimental groups consisted of 8–12 animals, assigned to the group randomly. Experiments were performed between 10 a.m. to 2 p.m. All described procedures were accepted by the Local Ethics Committee for Animal Experiments in Lublin and were in agreement with Directive 2010/63/EU on the protection of animals used for scientific purposes. Each mouse was used only once.

Chemical substances

Salbutamol (SALB; Polfa, Warsaw), butoxamine (Sigma, St. Louis, MO, USA), carbamazepine (CBZ; Polfa, Warsaw), phenytoin (DPH; Polfa, Warsaw), phenobarbital sodium salt (PB; Polfa, Warsaw), valproate magnesium salt (VPA; Polfa, Rzeszow) were used in this study. Butoxamine, PB and VPA were dissolved in sterile saline, whereas SALB, CBZ and DPH were suspended in 1% sterile solution of Tween 80 (Sigma, St. Louis, MO, USA). All analyzed drugs were injected intraperitoneally (*ip*) in a volume of 0.01 ml/g of body weight. To evaluate the influence of a single dose of SALB on seizure threshold in mice, the drug was given 15, 30, 60 and 120 min before the test. In other tests, SALB, butoxamine, CBZ and VPA were given 30 min, whereas PB 60 min and DPH 120 min before the test. The pretreatment times were based on the maximal activity of tested compounds according to the available literature and our previous experiments [33,34]. The times to the peak of maximum anticonvulsant effects for all AEDs were used as the reference times in all presented tests.

Animal experiments

Seizure activity

Electroconvulsions were delivered through ear clip electrodes and generated by a Hugo-Sachs stimulator (Hugo Sachs Rodent Shocker, type 221, Freiburg, Germany), producing alternating current (50 Hz, 500 V). Each stimulus lasted 0.2 s. Tonic hind limb extension was considered as the endpoint.

To evaluate seizure threshold, minimum four groups containing at least 8 animals were subjected to various shocks intensities. According to the amount of animals, in which tonic hind limb extension occurred after different shocks, the CS_{50} (median current strength) was calculated, as reported by Litchfield and Wilcoxon [35]. The CS_{50} value (in mA) represents the current strength needed to evoke tonic hind limb extension in 50% of the animals (shock intensities were chosen to get response in 10–25%, 30–50%, 50–70% or 70–90% of tested animals).

To analyze the AEDs' efficacy the MES-induced seizures were produced by a constant current of 25 mA intensity lasting for 0.2 s. The protective effect of AEDs was presented as ED_{50} (median effective dose, in mg/kg), which is a drug's dose protecting 50% of the tested animals from tonic hind limb extension. To calculate ED_{50} value minimum three groups consisting of at least 8 animals were used. Doses of the analyzed AEDs were selected to achieve protective effect in 10–25%, 30–70% or 70–90% of animals.

Experimental groups

Experiments were carried out after 1 and 7-day SALB treatment. Animals were divided into two groups:

1 Mice receiving *ip* SALB, butoxamine and AEDs in a single dose before the MES test.
2 Mice receiving everyday (between 8 a.m. and 10 a.m.) SALB *ip* for 7 days. On the 7th day the experimental procedures were performed in the same manner as in a single dose experiment. The control group received sterile saline or 1% sterile solution of Tween 80. On the last day of the experiment AEDs and butoxamine were administered in mice in appropriate times prior to the test.

Chimney test

The chimney test was used to analyze motor coordination in mice after SALB administration either alone or in combination with tested AEDs, according to Boissier et al. [36]. Mice were forced to climb backwards up a plastic tube (3 cm inner diameter, 25 cm

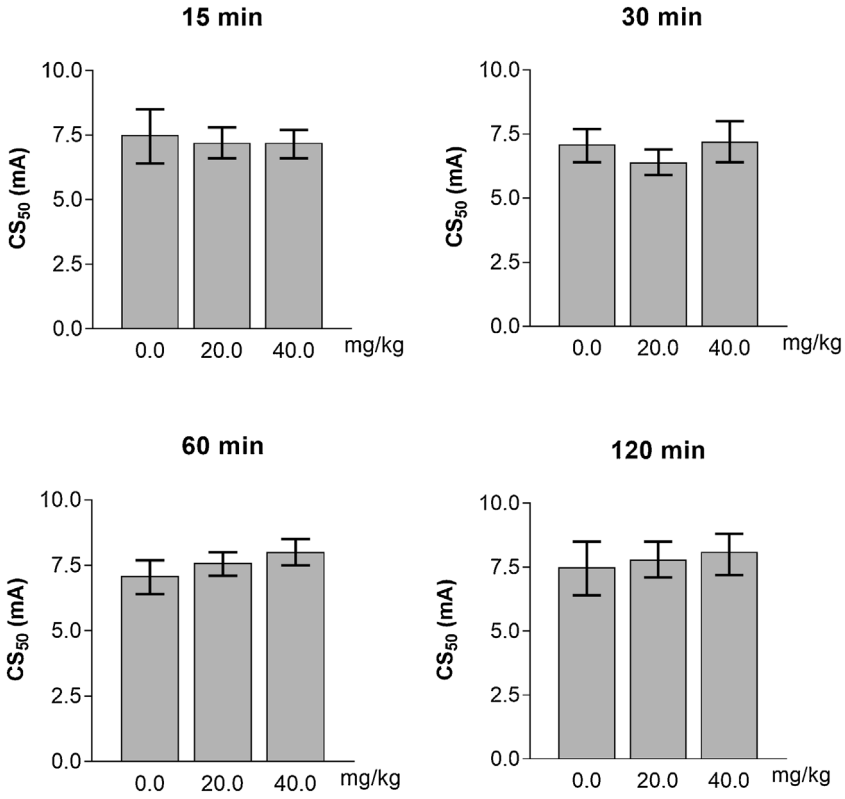

Fig. 1. Influence of a single dose of SALB on the threshold for MES-induced seizures in mice. Each examined group consisted of minimum of 8 animals. Presented data are CS_{50} values (with 95% confidence limits), calculated according to Litchfield and Wilcoxon [35].

length, with coarse surface). During experiments mice (minimum 8 animals in a group) received tested AEDs and SALB. The impaired motor coordination in mice was defined as inability of animals to climb up the tube within 60 s. After animals examination, the TD_{50} (median toxic dose) was calculated (in mg/kg), representing an AED dose causing motor coordination impairment in 50% of the tested animals, as shown by Litchfield and Wilcoxon [35].

Passive avoidance acquisition and retention tests

The step-through passive avoidance task was performed to analyze the effect of the examined substances on long-term memory acquisition in mice, according to Venault et al. [37].

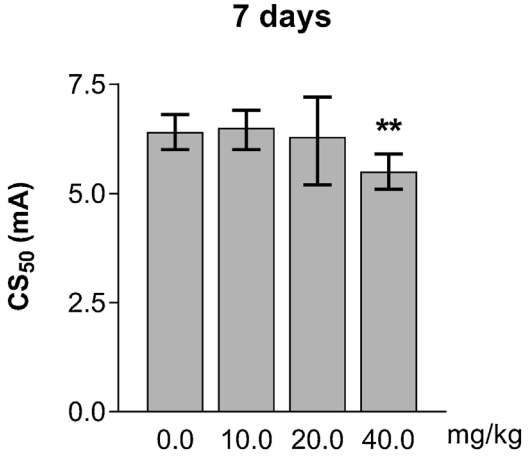

Fig. 2. Influence of SALB administered for 7 days on the threshold of MES-induced seizures in mice. Each examined group consisted of minimum of 8 animals. Presented data are CS_{50} values (with 95% confidence limits), calculated according to Litchfield and Wilcoxon [35], ** $p < 0.01$ *vs.* control.

Animals were not habituated to the passive avoidance device before the test. Each mice received an AED either singly or in combination with SALB one day before the training. Animals (12 in

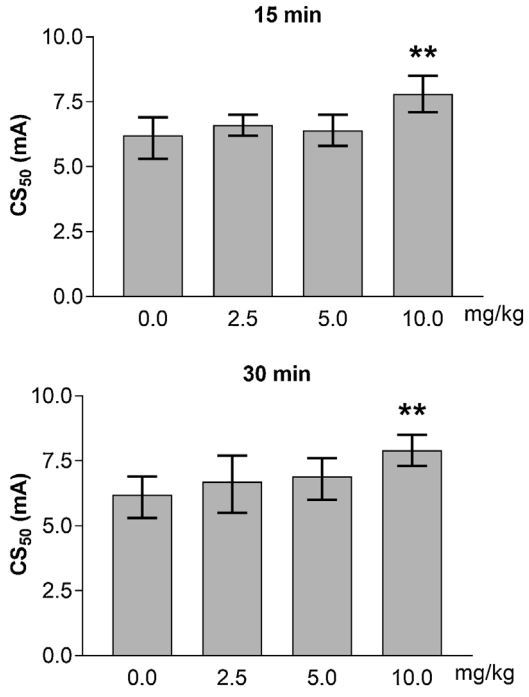

Fig. 3. Influence of butoxamine on seizure threshold in mice. Each examined group consisted of minimum of 8 animals. Butoxamine was given *ip* 15 min and 30 min prior to the test. Presented data are CS_{50} values (with 95% confidence limits), calculated according to Litchfield and Wilcoxon [35], ** $p < 0.01$ *vs.* control.

a group) after the respective pretreatment were transported into an illuminated box (10 × 13 × 15 cm) connected with second larger dark container (25 × 20 × 15 cm) equipped with an electric grid floor and observed for 120 s. Entering into the dark box was punished by an electric foot shock (0.6 mA lasting 2 s). Mice that did not enter the dark container within 60 s were excluded from the study. The next day (24 h later) animals not receiving drugs were placed again in the illuminated box and observed for 180 s. Mice avoiding dark container longer than 180 s were qualified as remembering the task. On the second day of testing no electric shocks were applied to mice. Retention times in the light box was presented as the medians with 25 and 75 percentiles. Tests were performed in the same time using the same drugs' doses as in the MES test.

Statistical analysis

CD_{50}, ED_{50} and TD_{50} values (with 95% confidence limits) were calculated according to Litchfield and Wilcoxon [35]. Results from passive avoidance test were analyzed by the nonparametric ANOVA Kruskal-Wallis test followed by *post hoc* Dunn's test.

Results

Effect of salbutamol single dose on the MES-induced seizure threshold

SALB at doses of 20 mg/kg and 40 mg/kg administered *ip* at 15, 30, 60 and 120 min before the test did not affect seizure threshold in the MES test in mice (Fig. 1).

Effect of 7 days administration of salbutamol on the MES-induced seizure threshold

After 7 days of administration SALB at a dose of 40 mg/kg decreased the seizure threshold from 6.4 mA to 5.5 mA ($p < 0.01$). On the 7th day of the experiment SALB was injected 30 min before the MES test (Fig. 2).

Effect of butoxamine on the threshold for MES-induced seizures

Butoxamine was injected at doses ranged from 2.5 to 10 mg/kg at 15 and 30 min before the MES test. Examined β_2-adrenergic receptor antagonist at a dose of 10 mg/kg increased the seizure threshold from 6.2 mA to 7.8 mA and 7.9 mA, respectively, in the MES test in mice, depending on time of administration (Fig. 3).

Effect of salbutamol on the antiepileptic efficacy of tested AEDs

SALB injected in a single dose or after 7 days of administration at a dose of 20 mg/kg did not change the ED_{50} of CBZ in the MES test in mice (Fig. 4A and B). Similarly, both single and repeated SALB administration did not affect VPA and DPH (Fig. 4A and B) ED_{50} values in the MES test in mice. On the contrary, the ED_{50} of PB was significantly higher after single (increased from 19.5 mg/kg to 26.9 mg/kg) and 7 days (changed from 22.3 mg/kg to 25.9 mg/kg) administration of SALB (Fig. 5A and B). Butoxamine (5 mg/kg) lowered the ED_{50} of PB from 26.9 mg/kg to 20 mg/kg after 1 day (Fig. 5A) and from 25.9 mg/kg to 21.8 mg/kg after 7 days of SALB administration (Fig. 5B), respectively. Butoxamine given alone at a dose of 5 mg/kg did not change the antiepileptic efficacy of PB (data not shown).

Effect of salbutamol on motor coordination impairment in mice after AEDs administration in the chimney test

Single (Fig. 6 A) and 7-day (Fig. 6B) administration of SALB (at the dose of 20 mg/kg) did not affect TD_{50} values of tested AEDs.

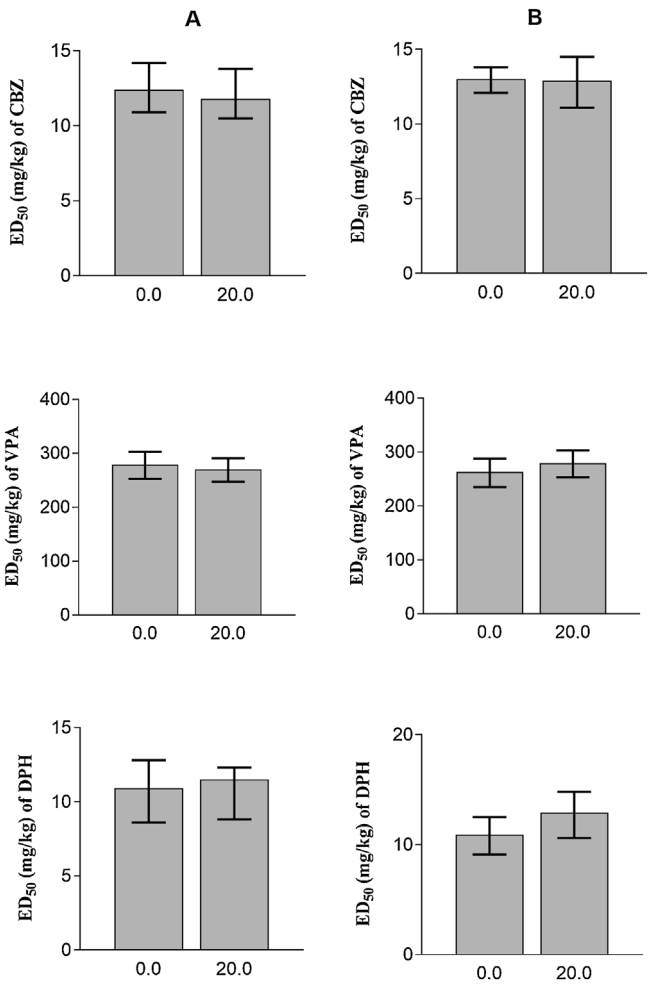

Fig. 4. Influence of single (A) and 7 days (B) administration of SALB on CBZ, VPA, DPH and PB antiepileptic activities in the MES test in mice. SALB was injected *ip* at a dose of 20 mg/kg. Each examined group consisted of minimum of 8 animals. Presented data are CS_{50} values (with 95% confidence limits), calculated according to Litchfield and Wilcoxon [35].

Effect of salbutamol on long-term memory in the passive avoidance test

SALB given as a single dose (Fig. 7) or after 7 days of administration (Fig. 8) had no effect on long-term memory in the passive avoidance test in mice. The AEDs (administered at doses corresponding to their ED_{50} values from the MES test) alone or in combination with SALB also did not affect long-term memory in mice (Figs. 7 and 8).

Discussion

The results of our study indicate that single administration of SALB (up to 40 mg/kg *ip*) did not affect the threshold in the MES-induced seizure test in mice. However SALB given for 7 days (20 mg/kg *ip*) significantly decreased the antiepileptic properties of PB (increased its ED_{50}), without any effect on the other AEDs tested. The observed effect was not a result of a pharmacokinetic interaction since the combination of SALB and PB did not change the free serum PB concentrations in mice (data not shown). Moreover, SALB did not have any influence on animals' motor coordination in the chimney test when given together with the examined AEDs. Additionally, SALB alone (20 mg/kg *ip*) and combined together with the AEDs (in doses equal to their ED_{50}

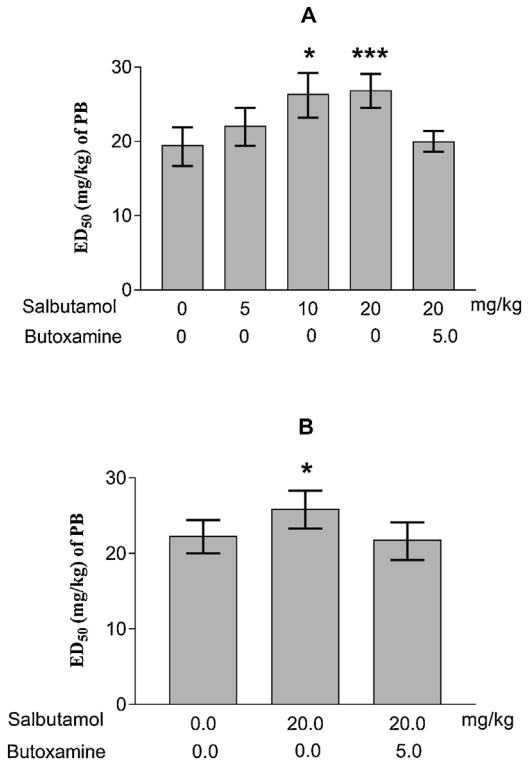

Fig. 5. Influence of single (A) and 7 days (B) administration of SALB on PB antiepileptic activity in the MES test in mice. SALB was injected *ip* at a dose of 20 mg/kg, butoxamine was given *ip* on 1st (A) or 7th (B) day of the experiment at a dose of 5 mg/kg. Each examined group consisted of minimum of 8 animals. Presented data are ED_{50} values (with 95% confidence limits), calculated according to Litchfield and Wilcoxon [35]. * $p < 0.05$ *vs.* control, *** $p < 0.001$ *vs.* control.

values) did not impair long-term memory in mice challenged in the passive avoidance test. Butoxamine, a selective β_2-adrenergic receptor antagonist, was administered in this study to confirm a possible involvement of β_2-adrenergic receptors in changing the PB's antiepileptic activity. However, the administration of butoxamine at the dose of 10 mg/kg *ip* increased seizure threshold in mice. Moreover, at the dose of 5 mg/kg *ip* butoxamine did not change the seizure threshold in mice as well as it did not affect the protective properties of PB. However, butoxamine at the dose of 5 mg/kg *ip* reversed effects of SALB (in both single and chronic administration), when β_2-adrenergic receptor agonist was given together with PB in mice.

The role of sympathetic nervous system in epilepsy pathogenesis has been widely discussed [20]. Effect of SALB on seizures induced by pilocarpine in rats was previously studied by Wamil and Kleinrok [38]. SALB at doses of 5, 7.5 or 10 mg/kg *ip* intensified convulsant effect of pilocarpine and shortened (at the dose of 5 mg/kg) the time to seizure onset. In the same study, propranolol, a nonselective β-adrenergic receptor antagonist at doses of 2, 3 and 5 mg/kg *ip* blocked effect of pilocarpine and SALB on seizure activity in rats [56]. Moreover, Łupina et al. analyzed the effect of β_2-adrenergic receptor agonists on aminophylline-induced seizures in mice [39]. Pretreatment with SALB, or other β_2-adrenergic receptor agonists fenoterol or terbutaline increased the risk of tonic-clonic seizures in mice.

Adversely, clonidine, an agonist of α_2-adrenergic receptors was reported to have antiepileptic potential in audiogenic seizures in DBA/2 mice [40], as well as in amygdala kindled [41], electrically [42] and chemically evoked seizures in rats [43]. However in the study made by Read et al. clonidine was shown to be weaker antiepileptic agent in comparison with β-adrenergic receptor antagonist atenolol in kainic induced seizures [44]. Similarly, an agonist of α_1-adrenergic receptors St 587 [2-(2-chloro-5-trifluoromethylphenylimino)imidazolidine] exhibited antiepileptic properties in amygdala kindled seizures in rats, however, it exerted no effect on threshold for electroshock- and pentylenetetrazole-induced seizures in rats [42]. Moreover, Gadie and Tulloch proven that this α_1-adrenergic receptor agonist even lowers seizure threshold for tonic phase of pentylenetetrazole-induced seizures in rats [45]. In addition, McIntyre and Wong reported that isoprenaline, a nonselective β-adrenergic receptor agonist may facilitate evoked and spontaneous activity of neurons in the pyriform cortex in rats *in vitro* [46]. On the other hand, isoprenaline injected intracerebroventricularly partially protected rats against electric seizures [47]. Interestingly, clenbuterol, a β_2-adrenergic receptor agonist raised the threshold for tonic phase in the MES–induced seizures in mice, the animal model of tonic–clonic seizures [48].

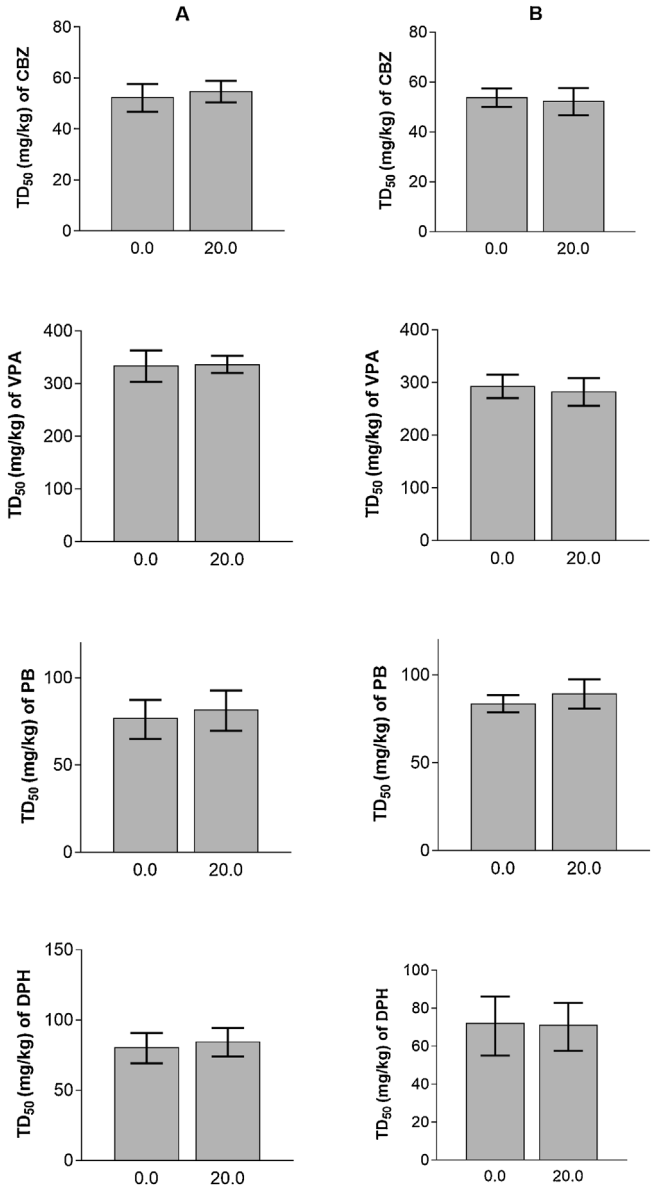

Fig. 6. Influence of single (A) and 7 days (B) administration of SALB on motor impairment in mice receiving CBZ, VPA, DPH or PB. SALB was injected *ip* at a dose of 20 mg/kg. Each examined group consisted of minimum of 8 animals. Presented data are TD_{50} values (with 95% confidence limits), calculated according to Litchfield and Wilcoxon [35].

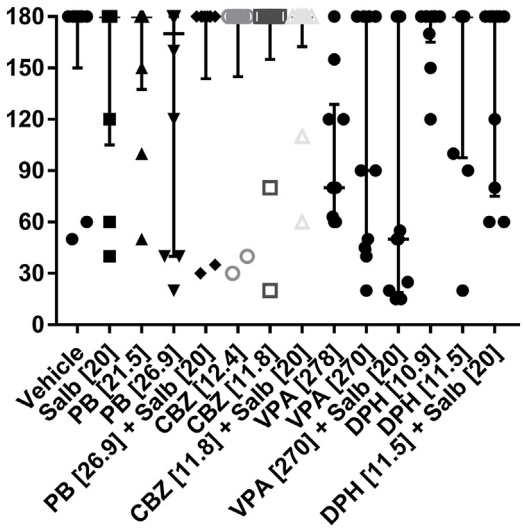

Fig. 7. Influence of a single SALB and AEDs administration on retention times in the passive avoidance task in mice. SALB, CBZ and VPA were injected *ip* 30 min, PB 60 min and DPH 120 min before the test. Presented data are medians with 25 and 75 percentiles of 12 determinations. The retention was shown as a time period expressed in seconds during which animals avoided the entrance into the dark compartment. Results were analyzed with the nonparametric ANOVA Kruskal-Wallis test followed by *post hoc* Dunn's test.

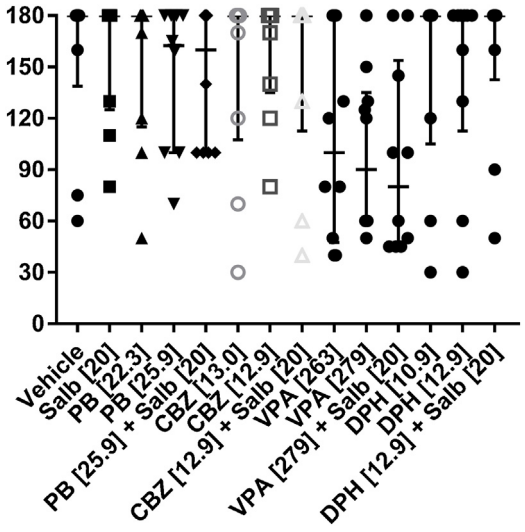

Fig. 8. Influence of 7-day SALB administration on retention times in the passive avoidance task in mice. SALB was administered *ip* for 7 days. On the 7th day salbutamol, CBZ and VPA were injected *ip* 30 min, PB 60 min and DPH 120 min before the test. Presented data are medians with 25 and 75 percentiles of 12 determinations. The retention was expressed as a time period (in seconds) during which animals avoided to enter the dark compartment. Results were analyzed with the nonparametric ANOVA Kruskal-Wallis test followed by *post hoc* Dunn's test.

The most broadly studied nonselective β-adrenergic receptor antagonist propranolol displayed antiepileptic effect in both, electroshock-induced [36] and chemically-induced seizures in rats [49]. Consistently, propranolol was reported to lower median effective doses (ED_{50}) of diazepam and VPA [50] and to potentiate the antiepileptic effect of glutamate receptors antagonists, MK-801 and GYKI 52466 in the maximal electroshock-induced seizure (MES) test in mice [51]. Similar protective properties were shown in experiments using other β-adrenergic receptor antagonists having weaker membrane stabilizing activity, *i.e.*, timolol [52] or pindolol [53]. Surprisingly, esmolol, a selective β_1-adrenergic receptor antagonist was reported to cause generalized convulsions in humans after intravenous infusion [54].

In presented study, butoxamine, a selective β_2-adrenergic receptor antagonist reversed negative impact of SALB on the antiepileptic efficacy of PB. However, when given alone butoxamine did not alter the ED_{50} of PB. This reveals a specific, depending on β_2-adrenergic receptor activity interaction between SALB and PB. Most studies concerning epilepsy pathogenesis analyzed the influence of drugs on β_1-adrenergic receptor activity. However, Louis et al. suggested that the antiepileptic effect of propranolol may result from blocking β_2-adrenergic receptors [55]. Furthermore, butoxamine and other β_2-adrenergic receptor antagonist, ICI 118.551, were reported to have protective effect in electrical [56] and isoniazid-induced convulsions [57] in rats. To highlight a possible role of noradrenergic neurotransmission in seizures treatment a locus coeruleus activation due to vagus nerve stimulation was also discussed [58].

Low antiepileptic activity of PB after SALB administration is not based on a pharmacokinetic interaction, since in our study SALB did not change free PB serum concentrations in tested mice. Still, a possible pharmacodynamic interaction between tested agents cannot be excluded. PB, CBZ and DPH are well known stimulators of cytochrome P-450 [59] and some β_2-adrenergic agonists are metabolized through cytochrome 3 A isoform [60], so AEDs influence on SALB's serum concentration cannot be ruled out. However, since SALB is not the substrate for common isoforms of cytochrome P 450 [61], a possible influence of AEDs on SALB liver metabolism rather should be excluded.

In conclusion, our study reveals that SALB lowers the antiepileptic efficacy of PB against MES-induced seizures in mice, whereas it has no effect on the protective properties of CBZ, DPH and VPA. A special attention should be paid to patients with epilepsy, who receive drugs activating β_2-adrenergic receptors.

Conflict of interest

There are no relevant disclosures or conflicts of interest.

Acknowledgement

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