Interaction between urethane and cannabinoid CB1 receptor agonist and antagonist in penicillin-induced epileptiform activity

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Previous experimental studies have shown that various anesthetics alter the effects of cannabinoid agonists and antagonists on the cardiac response to different stimuli. Since no data have shown an interaction between urethane and cannabinoid signaling in epilepsy, we examined the suitability of urethane with regard to testing the effects of a cannabinoid CB1 receptor agonist and an antagonist on penicillin-induced epileptiform activity in rats. Permanent screw electrodes for electrocorticographic (ECoG) recordings, and a permanent cannula for administration of the substances to the brain ventricles were placed into the cranium of rats. Epileptiform activity was induced by injection of penicillin through the cannula in conscious animal. The CB1 receptor agonist arachidonyl-2-chloroethylamide (ACEA; 7.5 μg) and the CB1 receptor antagonist [N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide] (AM-251; 0.25 μg) were administered intracerebroventricularly 30 minutes after the penicillin application in urethane-anesthetized and conscious animals. Urethane completely eliminated spontaneous ictal events in ECoG recordings and reduced the frequency and total amount of epileptiform activity. It did not alter either the proconvulsant effects of AM-251 or the anticonvulsant effects of ACEA on penicillin-induced epileptiform activity. The electrophysiological evidence suggests that there is no possible interaction between urethane and cannabinoid CB1 receptors in this experimental model of epilepsy.

Key words: epilepsy, cannabinoids, CB1 receptor, urethane, penicillin, conscious rat

INTRODUCTION

In experimental models of epilepsy, seizures have been induced in both anesthetized and conscious animals (Horn and Esseling 1993, Stringer et al. 2003, Kozan et al. 2006, Tutkun et al. 2015). Although a number of studies have induced seizures in anesthetized animals using various different anesthetic agents (Wu and Leung 2001, Stringer et al. 2003, Kozan et al. 2006, Arslan et al. 2013, 2014), these may interfere with brain function and may alter the susceptibility of the brain to convulsant drugs (Hunfeld et al. 2013). Previous investigations regarding the effects of anesthetic agents on epilepsy have been inconclusive. Some studies have shown that urethane is not an ideal anesthetic agent because it mimics normal sleep, with active and quiet stages on EEG profiles, and inhibits epileptic activity (Heltovics et al. 1995, Clement et al. 2008, Hunfeld et al. 2013), while others have reported that urethane is a suitable anesthetic to describe the firing properties of neurons in epileptic activity (Hara and Harris 2002, García-Hernández et al. 2010). Although little information is available regarding the actions of urethane at the cellular and synaptic levels (Hunfeld et al. 2013), it has been reported that it has little influence on the neuronal activity of a wide variety of central nervous system areas, including the cerebral cortex (Angel and Gratton 1982). Brožičková and Otáhal (2013) suggested that urethane is suitable for future experiments in models of epilepsy.

On the other hand, cannabinoid systems appear to regulate seizure activity in the brain through the activation of cannabinoid CB1 receptors (Wallace et al. 2003, Deshpande et al. 2007, Kozan et al. 2009, Arslan et al. 2014, Agar 2015). A large series of cannabinoid analogues have been tested in various experimental models of epilepsy, including a rat pilocarpine model (Falenski et al. 2007), the maximal electroshock model for grand-mal seizures (Wallace et al. 2001), a pentylentetrazole model of myoclonic seizures in mice (Gholizadeh et al. 2007) and a penicillin-induced model of epileptiform activity in the rat (Kozan et al. 2009, Arslan et al. 2014). The majority of these studies have been conducted under anesthesia, which may interact with other drugs that affect epileptic activity, such as cannabinoids. It has been shown that
cannabis is capable of prolonging both pentobarbitone and ether anesthesia by a direct depressant effect that is unrelated to any effect on the central 5-hydroxytryptamine or catecholamine neurons of mice (Chesher et al. 1974). In addition, administration of the selective CB2 receptor agonist, AM-1241, with tramadol improved the antinociceptive effects and immune responses of cannabinoids in a rat incisional pain model (Stachtiari et al. 2014). It has also been shown that urethane abolishes the H₂ receptor-mediated vascular response in pithed rats and attenuates the CB1 receptor-mediated cardiac response to a far greater extent than pentobarbitone (Kurz et al. 2009). Therefore, the choice of anesthetic may play an important role in the outcome of investigations (Kurz et al. 2009, Hunfeld et al. 2013), and it is always prudent to be aware of the potential interactions between the cannabinoid system and anesthetic agents in vivo studies, including those of epilepsy. To our knowledge, no previous studies have compared the effects of cannabinoid CB1-receptor agonist and antagonist agents in an experimental model of anesthetized and conscious animals. Therefore, in the present study, we compared the effects of the cannabinoid CB1 receptor agonist, arachidonyl-2-chloroethylamide (ACEA), and the antagonist [N-(piperidin-1-yl)-5-[(4-i odophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3 carboxamid e](AM-251) on conscious rats and urethane-anesthetized animals. The relationship between epileptiform discharges and the dose of convulsant agent (penicillin) was also addressed in conscious rats.

**METHODS**

**Animals**

A total of 77 male specific-pathogen-free Wistar rats were purchased from the Animal House of Ondokuz Mayis University, Samsun, Turkey. All animals, weighing 240–280 g, were maintained in a temperature-controlled environment in a 12-h light/dark cycle, with free access to tap water and standard laboratory food. The local ethics committee approved all experimental procedures (2009/42). The rats were assigned to experiments and groups, as follows: In the first set of experiments, conducted in anesthetized groups, group one received normal saline-dimethylsulfoxide (DMSO); group two received 500 units penicillin; group three received 500 units penicillin+DMSO (final solution DMSO/saline: 3/7); and group four received 500 units penicillin+0.25 μg AM-251; group five 500 units penicillin+7.5 μg ACEA. In the second set of experiments, conducted in conscious animals, group six received normal saline+DMSO; group seven received 500 units penicillin; group eight received 300 units penicillin; group nine received 300 units penicillin+DMSO; group 10 received 300 units penicillin+0.25 μg AM-251; and group 11 received 300 units penicillin+7.5 μg ACEA. Each group was composed of seven rats.

**Drugs and drug administration**

Sterile physiological normal saline, penicillin G potassium (I.E. Ulagay, Turkey), AM-251 and ACEA (Sigma Chemical Co., St. Louis, MO, USA) were used. Penicillin G potassium was dissolved in normal saline, and 2.5 μl was injected into the lateral ventricle of rats in the normal saline group.

AM-251 and ACEA were dissolved in dimethylsulfoxide (DMSO) with sterile physiological saline (final solution DMSO/saline 3:7 volume/volume, respectively) and 1 μl of the required doses was administered intracerebroventricularly (i.c.v.). The drug doses were determined in accordance with previous studies (Aslan et al. 2009, 2010).

In the first set of experiments, the i.c.v. injections were performed according to the method reported by Arslan and others (2014). In brief, a microsyringe was inserted into the left lateral ventricle of each rat through stereotaxic apparatus. The drug solutions were injected at an infusion rate of 0.5 μl/min, using a Hamilton microsyringe (type 701N; Aldrich, Milwaukee, WI, USA), and the needle remained in place for an additional minute to prevent backflow of the drug. The epileptic focus was produced via an injection of 500 units of penicillin G potassium in a volume of 2.5 μl into the lateral ventricle. The doses of ACEA (7.5 μg) and AM-251 (0.25 μg) were administered 30 minutes after the i.c.v. application of penicillin (Arslan et al. 2013).

In the second set of experiments, the animals were gently hand-restrained for the i.c.v. drug administration, and infusions were made using an injector cannula connected by a polyethylene tube to a 10 ml Hamilton (type 701N) syringe (infusion rate, 0.5μl/min). Subsequently, penicillin G potassium (300 IU) and the doses of ACEA (7.5 μg, i.c.v.) and AM-251 (0.25 μg) were injected into the left lateral cerebral ventricle. After recording basal activity for 10 minutes, 2.5 μl penicillin (300 IU) was injected into the lateral cerebral ventricle through a cannula. Epileptiform activity was started after approximately 2 minutes. The remaining substances were administered to the lateral cerebral ventricle 30 minutes after penicillin-induced epileptiform activity.

**Placement of electrodes for electrocorticography recordings**

In the first set of experiments, the animals were anesthetized with urethane (dissolved in distilled water,
1.25 g/kg, intraperitoneally) and placed in rat stereotaxic apparatus. With stereotaxic guidance, two screw electrodes were placed over the left somatomotor cortex, and a ground lead was positioned over the nasal sinus. Two bipolar Ag–AgCl ball electrodes were placed over the somatomotor cortex of the left hemisphere (Kozan et al. 2007, Arslan et al. 2014). In the second set of experiments, all animals were anesthetized prior to surgery using ketamine (50 mg/kg) (Alfamine®, Alfasan International B.V. Netherlands) and xylazine (7.5 mg/kg) (Alfazyne®, Alfasan International B.V. Netherlands) intraperitoneally. The animals were then placed in stereotaxic apparatus. The skin and subcutaneous tissue were removed from the cranium, and the skin was folded back with retractors. Firstly, a small burr hole was drilled with a hand drill, then an external i.c.v. cannula was stereotactically implanted into the left lateral cerebral ventricle, taking bregma as the reference point (coordinates AP: −1.0 mm, LL: 1.5 mm, DV: −3.2 mm) (Kozan et al. 2007, Arslan et al. 2014). For the tripolar recording electrodes (Plastic products company, MS 333/2A), three burr holes were drilled and the tips of the electrodes were inserted.

![Diagram of electrode placement](image-url)

Fig. 1. Urethane-anesthetized groups: (A) The intracerebroventricular (i.c.v.) injection of penicillin (500 IU) induced epileptiform activity on ECoG (79.1±6.9 spikes/min; 984±186 µV). (B) Administration of the CB1 receptor antagonist AM-251 (0.25 μg, i.c.v.) resulted in a marked increase in the frequency of penicillin-induced epileptiform activity within 30 minutes of AM-251 injection. (C) Administration of the CB1 receptor agonist ACEA (7.5 µg, i.c.v.) significantly decreased the mean frequency of epileptiform activity within 60 minutes of ACEA injection. (D) DMSO (DMSO/saline 3:7 volume/volume, 1 μl, i.c.v.) injection did not alter the baseline activity. Conscious groups: (E) The intracerebroventricular (i.c.v.) injection of penicillin (300 IU) induced epileptiform activity on ECoG (162.2±16.4 spikes/min; 948±106 µV). (F) Administration of the CB1 receptor antagonist AM-251 (0.25 μg, i.c.v.) led to a marked increase in the frequency of penicillin-induced epileptiform activity within 30 minutes of AM-251 injection. (G) Administration of the CB1 receptor agonist ACEA (7.5 µg, i.c.v.) significantly decreased the mean frequency of epileptiform activity within 70 minutes of ACEA injection. (H) DMSO (DMSO/saline 3:7 volume/volume, 1 μl, i.c.v.) injection did not alter the baseline activity. Representative ECoGs are presented for the 100 minutes after penicillin administration.
to the holes (First AP: +4.0 mm, LL: 3.0 mm; second AP: -4.0 mm, LL: 3.0 mm; third AP: -4.0 mm, RL: 3.0 mm). The electrodes and external cannula were fixed using cold dental acrylic. The rats were allowed at least a week to recover from the surgery, and were transferred to an observation cage (40×40×40 cm). Fully conscious animals were connected to a computerized electrocorticographic (ECoG) recording system by an isolated flexible cable, and ECoG activity was continuously monitored on an eight-channel recorder (PowerLab, 8/SP, AD Instruments, Castle Hill, NSW, Australia). The frequency and amplitude of the epileptiform ECoG activity was analyzed off-line. Value % was calculated for each 10 minutes. At the end of the experiments, the position of the cannula was visually confirmed by 2% methylene blue infusion through the i.c.v. cannula after the animals were given a lethal dose of anesthetic.

In order to determine behavioral activity, the animals were observed every 15 minutes according to the seizure severity scale (Fischer and Kittner 1998): (Stage 0) no convulsion; (Stage 0.5) weak head-nodding; (Stage 1) ear, face and eyelid twitching; (Stage 1.5) mild forelimb clonic activity; (Stage 2) myoclonic body jerks, clonic forelimb convulsions without rearing; (Stage 2.5) partial rearing and rapid clonic seizures of forelimb; (Stage 3) powerful bilateral forelimb clonus with complete rearing (≥10 s); (Stage 3.5) rearing and falling with intense bilateral forelimb clonus; (Stage 4) generalized clonic seizures with rearing and falling down episodes, or jumps; (Stage 4.5) generalized clonic-tonic seizures with failure of righting reflex; (Stage 5) generalized clonic-tonic seizures and status epilepticus (≥2 min).

**Statistical analysis**

All results are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were made using GraphPad Instat (v3.06) software (GraphPad Software, San Diego, CA, USA). The normality of the data was tested using the Shapiro-Wilk test before analyses. After verifying that the data obtained from electrophysiological recordings were normally distributed, one-way analysis of variance and Tukey-Kramer post hoc tests for multiple comparisons were performed. For all statistical tests, p<0.05 was considered statistically significant.

**RESULTS**

The effect of urethane on penicillin-induced epileptiform activity

A single i.c.v. injection of penicillin (500 units) induced epileptiform activity approximately 2 minutes after injection; the activity reached a constant level by 30 minutes after penicillin administration and persisted
for approximately 3 hours in anesthetized rats. The means of the spike frequency and amplitude of the epileptiform activity were 79.1±6.9 spikes/min and 984±186 µV, respectively, in the anesthetized group (Fig. 1A).

In conscious rats, a dose of 500 IU penicillin was administered to the lateral ventricle to induce epileptiform activity. However, all animals died within 10–30 minutes due to severe seizures. A single dose of 300 IU penicillin was then administered to the lateral ventricle, which induced epileptiform activity approximately 2 minutes after injection; the activity reached a constant level in 30 minutes after penicillin administration and persisted for approximately 3 hours. Penicillin (300 IU) resulted in two different epileptic activity events, interictal and ictal events, in the ECoG of conscious rats. The means of the spike frequency and amplitude of the ictal and interictal epileptiform activity events were 162.2±16.4 spikes/min and 948±106 µV, respectively (Fig. 1E).

**Interaction between urethane and the cannabinoid CB1 receptor agonist and antagonist in penicillin-induced epileptiform activity**

It has been found that the most effective doses of the cannabinoid CB1 receptor agonist, ACEA (7.5 µg), and antagonist, AM-251 (0.25 µg), in urethane-anesthetized rats (Kozan et al. 2009) and conscious rats are those that are administered i.c.v. 30 minutes after penicillin injection. In the present study, AM-251 increased the mean frequency of penicillin-induced epileptiform activity in anesthetized rats in the 30 minutes after AM-251 injection without changing the amplitude (Fig. 2A). The means of the spike frequency and amplitude of the epileptiform activity were 122.1±7.5 spikes/min and 1001±113 µV, respectively, in the 100 minutes after AM-251 injection (Fig. 1B). AM-251 administration also led to the development of status epilepticus-like activity in anesthetized rats (Fig. 1B). The same dose of AM-251 increased the mean frequency of penicillin-induced epileptiform activity in conscious rats in the 30 minutes after the AM-251 injection, without changing the amplitude (Fig. 2B). In these rats, the means of the spike frequency and amplitude of ictal and interictal epileptiform activity were 240.5±19.3 spikes/min and 887±93 µV, respectively, in the 100 minutes after AM-251 injection (Fig. 1F). ACEA administration significantly decreased the mean frequency of penicillin-induced epileptiform activity in anesthetized rats in the 60 minutes after ACEA injection, without changing the amplitude (Fig. 2A). The means of the spike frequency and amplitude of the epileptiform activity were 35.1±4.6 spikes/min and 873±104 µV, respectively, in the 100 minutes after ACEA injection (Fig. 1C). The administration of ACEA significantly decreased the mean frequency of penicillin-induced epileptiform activity in conscious rats in the 70 minutes after ACEA injection, without changing the amplitude (Fig. 2B). The means of the spike frequency and amplitude of ictal and interictal epileptiform activity were 96.1±9.8 spikes/min and 768±98 µV, respectively, in the 100 minutes after ACEA injection (Fig. 1G).

![Fig. 3. The effects of intracerebroventricular (i.c.v.) administration of the CB1 receptor antagonist, AM-251, and the agonist, ACEA, on the total number of spikes in urethane-anesthetized and conscious rats. (A) AM-251 (0.25 µg, i.c.v.) increased the total number of spikes by 168%, whereas the administration of ACEA significantly decreased the total number of spikes by 58% in urethane-anesthetized rats. (B) AM-251 (0.25 µg, i.c.v.) increased the total number of spikes by 159%, whereas the administration of ACEA significantly decreased the total number of spikes to 63% in conscious rats. *p<0.05; **p<0.01; ***p<0.001 indicate significant differences compared to the control group.](image-url)
The total number of spikes observed in anesthetized rats was increased up to 168% in the presence of AM-251, whereas it decreased to 58% in the presence of ACEA (Fig. 3A). In conscious rats, the total number of spikes observed an increase to 159% in the presence of AM-251, but decreased to 63% in the presence of ACEA, compared to the control group (Fig. 3B). The means of interictal and ictal spike activity observed in conscious rats increased to 147% and 167%, respectively, in the presence of AM-251, but was significantly decreased to 65% and 62%, respectively, with ACEA compared to the control group (Fig. 4). The administration of AM-251 also increased both the severity of seizure scale and the total amount of ictal activity in conscious rats to 134% and 179%, respectively, whereas they were decreased to 68% and 57%, respectively, by ACEA compared to the control group (Fig. 5).

Intracerebroventricular injection of normal saline (2.5 µl)+DMSO (DMSO/saline 3:7 volume/volume, respectively, 2.5 µl) did not statistically affect the mean frequency of penicillin-induced epileptiform activity compared with the control group in anesthetized rats, as shown previously (Arslan et al. 2014) or in conscious rats. None of the drugs or vehicles resulted in any change in baseline activity when administered alone in both anesthetized and conscious animals (Figs 1D, 1H).

**DISCUSSION**

The aim of the present study was to search for an interaction between urethane and the effects of the CB1 receptor agonist ACEA, and the antagonist AM-251, on penicillin-induced epileptiform activity in rats. Urethane has been widely used as an anesthetic in combination with a number of different chemicals in experimental epilepsy studies (Rose 1979, Campbell and Holmes 1984, Largo et al. 1997, Cakil et al. 2011, Erfanparast and Tamaddonfard 2015). The choice of anesthetic is crucial, as it may interact with test drugs with regard to some of the parameters studied in in vivo experiments (Chesher et al. 1974, Kurz et al. 2009, Hunfeld et al. 2013). The present study shows that the doses of penicillin required to induce epileptiform activity were higher in urethane-anesthetized rats than in conscious rats, and that there was no interaction between urethane and the cannabinoid CB1 receptor agonist, ACEA, and the antagonist, AM-251, regarding their effects on penicillin-induced epileptiform activity.

**The effect of urethane on penicillin-induced epileptiform activity**

Urethane is a commonly used as an anesthetic in experimental epilepsy models because it provides a stable and prolonged level of anesthesia. It has been shown that intracortical injections of 1.25 µg doses of kainic acid result in epileptiform activities approximately 14 min after injection in all paralyzed animals (Hunfeld et al. 2013). The same dose of kainic acid led to epileptiform activity in only one of four urethane-anesthetized rats; higher doses of kainic acid (between 3.75 µg and 10 µg)
were required to induce epileptiform discharges in the remaining three rats (Hunfeld et al. 2013). In other models of experimental epilepsy, doses of picrotoxin lower than 2.4 mg/kg (intravenously administered) did not evoke any signs of phrenic or hypoglossal motor output in anesthetized rats, whereas convulsive symptoms appeared at a cumulative dose of 2.4 mg/kg picrotoxin (Budzińska 2004). Willoughby and others (1995) suggested that a low dose of picrotoxin (0.9–2.0 mg/kg) was required to induce seizures in conscious rats. In accordance with previous investigations, the present study shows that the doses needed to induce epileptiform activity were higher in urethane-anesthetized rats than in conscious rats, as 500 units of penicillin administered in the course of previous experiments caused death, due to severe seizures. Furthermore, the total number and frequency of epileptiform spikes was much higher in conscious rats compared to urethane-anesthetized rats, despite the low dose of penicillin used to induce epileptic activity in the conscious animals. Although interictal epileptic activity also reflects a state of neuronal excitability related to seizure frequency (Ebus et al. 2004), urethane depressed the ictal events in ECoG during electrographic seizures in the anesthetized rats compared to the conscious rats in the present study.

The precise mechanism of urethane is not yet understood, and the mechanism of the inhibitory effect of urethane on epileptiform activity remains uncertain. It has been shown that urethane potentiates the functions of neuronal nicotinic acetylcholine, GABAA, and glycine receptors, and inhibits N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors in a concentration-dependent manner in Xenopus oocytes (Hara and Harris 2002). Hara and Harris (2002) also suggested that the anesthetic concentration of urethane can modulate the activities of all receptors tested. According to the GABA hypothesis of epilepsy, insufficient GABAergic inhibition and an increase in glutamatergic excitation are two reasons for the initiation and spread of epileptic seizures (Corda et al. 1991, Löscher 1993, Meldrum 1995). Penicillin exerts its proconvulsant effect by inhibiting GABA-gated chloride ion influx. There is also a link between NMDA receptor activity and the potentiation of GABAergic transmission in the experimental epilepsy model, including penicillin (Tsuda et al. 1997, Lu et al. 1998). It is therefore logical to expect reduced epileptiform activity in the instances of urethane use in the present study.

Interaction between urethane and the cannabinoid CB1 receptor agonist and antagonist in penicillin-induced epileptiform activity

In the present study, the CB1 receptor antagonist AM-251 significantly increased the frequency of epileptiform activity up to 168% and 159% in urethane-anesthetized and conscious rats, respectively. AM-251 also led to an increase in the total number of spikes in both groups. The CB1 receptor agonist ACEA significantly decreased the frequency of epileptiform activity in both urethane-anesthetized and conscious rats, and also decreased the total amount of epileptiform spikes to 58% and 63% in urethane-anesthetized and conscious rats, respectively.
CONCLUSIONS

The results of present study show that the doses of substance required to induce epileptiform activity were higher in urethane-anesthetized rats than in conscious rats. In addition, urethane completely eliminated penicillin-induced ictal events in ECoG recordings, and reduced the frequency and total amount of epileptiform activity. Urethane did not alter the effect of the CB1 cannabinoid antagonist AM-251 or the agonist ACEA on penicillin-induced epileptiform activity, suggesting no interaction between urethane and the cannabinoid CB1 receptor agonist and antagonist in the experimental epilepsy model. Therefore, it can be suggested that urethane is suitable for maintaining anesthesia during electrophysiological recordings, at least for studying the role of cannabinoids on the experimental model of epilepsy used in the present study.

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References


