Anandamide Induced Anti-Convulsion in an In Vitro Model of Epilepsy

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ABSTRACT. In both in vitro and in vivo models of epilepsy, cannabinoids had anti-convulsant properties, which have been shown to be mediated through activation of central cannabinoid type 1 (CB₁) receptors. The current study used 24 adult Sprague-Dawely rats to investigate the effects of endogenously occurring cannabinoids (endocannabinoids) on epileptiform activity induced by picrotoxin. Extracellular recordings were made from stratum pyramidale of the CA₁ region of hippocampal slices maintained in a submersion type recording chamber. Stimulation with single pulses evoked population spikes of approximately equal amplitude. Using single pulse stimulation, perfusion of 0.5 µM picrotoxin caused a small increase in the amplitude of the first population spike, and caused epilepsy by introducing a second or multiple population spikes. In the presence of picrotoxin, anandamide reduced the amplitude of both the first population spike (PS1) and the second population spike (PS2), thus reducing the epilepsy. The CB₁ receptor antagonist, AM 281 (500 nM) had no effect on responses recorded in the presence of picrotoxin, but totally blocked the effect of subsequently perfused anandamide. The results showed that anandamide caused an anti-convulsion effect. Furthermore, these results implicate the cannabinoid CB₁ receptor as a major endogenous site of seizure modulation.

Keywords: Anandamide, Endogenous cannabinoids, Anti-convulsion, Hippocampal slice.
Introduction

Epilepsy is one of the most common neurological conditions and is characterised by spontaneous recurrent seizures\cite{1}. Understanding the pathophysiology of seizure initiation and termination would have important implications for our ability to manage seizure disorders and for the potential development of novel anti-epileptic agents. Previous research in our laboratory\cite{2} and others\cite{3-5} have shown that cannabinoid compounds such as WIN55,212-2 are anti-epileptic compounds in both *in vitro* and *in vivo* studies. We further demonstrated that the anti-epileptic effect of cannabinoids was mediated through the central cannabinoid CB$_1$ receptors\cite{2}.

Two different cannabinoid (CB$_1$ and CB$_2$) receptors have been identified, so far, which were cloned in 1990 and 1993, respectively\cite{6}. CB$_1$ is the type preferentially expressed in the brain and is known to mediate the psychoactive effect of cannabinoids\cite{7}. The distribution of CB$_1$ receptors is not homogenous in the brain, CB$_1$ receptors were found to be very abundant in the hippocampus, neocortical area, and the limbic system, the areas believed to modulate seizure activity\cite{8}. The discovery of cannabinoid receptors was followed in 1992 and 1995 by the demonstration of the existence of endogenous cannabinoid receptor agonists\cite{9}. The most important of these are arachidonylethanolamide synthase (anandamide synthase) and 2-arachidonylglycerol where there is evidence that both can serve as neuromodulators or neurotransmitters\cite{10}. As our previous study has shown an anti-epileptic effect of the cannabinoid CB$_1$ agonist WIN55,212-2, the next logical step is to investigate the role of endogenous cannabinoids in the anti-epileptic scenario, which is the aim of the current study.

Materials and Methods

The study was conducted in the Department of Biomedical Sciences, Institute of Medical Sciences, Aberdeen University, Aberdeen, UK. Twenty-four young adult Sprague-Dawley rats aged from 5- to 7- weeks were used in the current study. After general anesthesia with halothane, they were sacrificed and the brain was removed from the skull and submerged in oxygenated cold (under 4°C) artificial cerebrospinal fluid (aCSF). The hippocampus was dissected out and chopped transversely on a McIlwain tissue chopper forming slices 400 µm thick. The slices were placed onto a moist filter paper in a Petri dish and maintained in a well-oxygenated and humidified chamber. After at least one hour, slices were transferred to a submersion-type recording chamber which was continuously perfused with aCSF at a rate of 1.5 ml/min. The temperature was maintained between 28-30°C. A bipolar stimulating electrode was used to stimulate the Schaffer collateral commissural fibres and evoked population spikes were recorded from the cell body layer of the CA1 region of the hippocampus.
using a glass capillary microelectrode filled with 3M sodium chloride (NaCl). Half-maximal population spikes were then evoked at 30 second intervals until a stable baseline of at least 30 min was established. Data was stored and analysed using the LTP program[11].

Drugs were applied by addition to the perfusion medium. Stock solutions of the endocannabinoid CB₁ receptor agonists (anandamide) were made up in alcohol and stored at 4°C. When required they were mixed with Tween 80 (two parts Tween 80 to one part of anandamide) and the ethanol was evaporated by using steam of nitrogen gas. Saline was then added in aliquots of 0.05 ml and the solution diluted with aCSF to obtain the required concentration. AM281 (the cannabinoid CB₁ receptor antagonist) was made up as a 10 mM stock solution in dimethyl sulfoxide (DMSO) and diluted in aCSF as required.

Epilepsy was induced by using the convulsant poisonous plant derivative 'picrotoxin', which is a non-competitive GABAA receptor antagonist. Picrotoxin has been widely used to induce epilepsy in the in vitro preparations[12]. The response recorded after the application of picrotoxin showed multiple population spikes which could be named as PS1, PS2, etc...(Fig. 1).

![Fig. 1. An example of a synaptic response recorded from the CA1 region of the hippocampal slice showing multiple population spikes after application of picrotoxin (500 nM). OS1 is the first population spike and the upward arrow indicates the amplitude (height) of the population spike in millivolt (mV). OS2 is the second population spike and the upward arrow indicates the amplitude (height) of the population spike in mV. Inset shows the time scale of the synaptic response.](image_url)

In all cases, statistical analysis was performed using the INSTAT program to measure significance using the paired "Student’s" t test (t test). Each slice included in the results came from a different rat. A P value of less than 0.05 was considered statistically significant. Anandamide, Tween 80 and AM281 were obtained from Tocris (Bristol, UK).
Results

1. Picrotoxin induced convulsions in the rat hippocampal slice: After recording a steady baseline for at least 30 minutes, perfusion of picrotoxin (500 nM) for 30 min caused epilepsy by introducing a second population spike (PS2) and increasing the amplitude of the first population spike (PS1) from the mean baseline value of 64.3 ± 5% to 78.5 ± 3% (n=5, Fig. 2).

2. Anandamide is an anticonvulsant in the in vitro model of epilepsy: After a steady baseline has been recorded for at least 30 minutes, picrotoxin (500 nM) was perfused until a PS2 was introduced and had reached a steady baseline. The perfusion of picrotoxin increased the amplitudes of PS1 and PS2. Anandamide (10 µM) was then perfused for 30 min. Perfusion of anandamide (10 µM) reduced the amplitude of PS1 to 53.4 ± 7% of the picrotoxin baseline. The amplitude of PS2 was reduced to 42.6 ± 6% of the picrotoxin baseline (n=8, Fig. 2). Anandamide therefore showed a strong anticonvulsant effect.

**Fig. 2.** Perfusion of anandamide (10 µM) for 30 minutes caused a significant reduction (P<0.05) of the amplitude of PS1 (A) and PS2 (B) in slices where epileptiform activity had been induced by perfusion of picrotoxin (500 nM). Data were presented as mean amplitude (% of picrotoxin baseline).
3. Anandamide’s anticonvulsant effect is mediated by cannabinoid's CB₁ receptor activation: A pre-treatment dose of the cannabinoid CB₁ receptor antagonist, AM 281 (500 nM) was used to test if the effect of anandamide was mediated through the central cannabinoid CB₁ receptors or not. Stable control response was obtained prior to the perfusion of 500 nM picrotoxin. Once a stable PS2 was obtained, the CB₁ receptor antagonist AM 281 (500 nM) was perfused for 30 min. This was followed by a perfusion of anandamide (10 µM) for 30 min. AM 281 alone did not affect the amplitude of the PS1 and PS2. After 30 min perfusion of AM 281, the amplitude of PS1 was 95 ± 4% of the picrotoxin baseline (i.e., no significant change). AM 281 markedly reduced the effect of the subsequently perfused anandamide (10 µM). The perfusion of anandamide (10 µM) for 30 min resulted in a very small reduction of the amplitudes of PS1 and PS2 (94 ± 8% and 92 ± 9%, respectively) which were not statistically significant (n=6, p < 0.05, Fig. 3).

**Fig. 3.** Perfusion of CB₁ receptor antagonist AM 281 (500 nM) for 30 min had no significant effect (P < 0.05) on the amplitude of PS1 (A) and PS2 (B) in slices where epileptiform activity had been induced by perfusion of picrotoxin (500 nM). However, AM 281 totally blocked the effect of subsequently perfused anandamide (10 µM) for 30 min. Data were presented as mean amplitude (% of picrotoxin baseline).
4. The effect of anandamide was not due to the drug vehicle Tween 80: The drug vehicle Tween 80, used to disperse anandamide, had no effect on population spikes. Perfusion of Tween 80 alone for 30 min at a concentration equivalent to that used to disperse 10 µM anandamide had no effect on either the amplitude of PS1 or the amplitude of PS2. After 30 min of Tween 80 perfusion, the amplitudes of PS1 and PS2 were 98 ± 11% and 102 ± 6% of the original baseline respectively (n=5, p<0.05, Fig. 4).

![Graph A](image1.png)

![Graph B](image2.png)

**FIG. 4.** Perfusion of the drug vehicle Tween 80, used to disperse anandamide, for 30 min has no significant effect ($P \leq 0.05$) on the amplitude of PS1 (A) and PS2 (B) in slices where epileptiform activity had been induced by perfusion of picrotoxin (500 nM). Data were presented as mean amplitude (% of picrotoxin baseline).

**Discussion**

The results of the current study demonstrate that anandamide, an endogenous cannabinoid, is a potent anticonvulsant in an *in vitro* model. The data also demonstrate that this anticonvulsant effect is mediated by activation of the cannabinoid CB$_1$ receptor.
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Anandamide was the first putative endogenous ligand for cannabinoid receptors to be identified\cite{13}, therefore attention has focussed on whether anandamide may also act physiologically to induce some useful medical effects. Anandamide is an eicosanoid that belongs to a class of fatty-acid derivatives of N-arachidonyl-phosphatidylethanolamine. The compound is reported to be synthesized “on-demand” by phospholipase-D in a depolarization and calcium-dependent manner\cite{14}. Previous researchers have shown that elevated intracellular calcium accompanies seizure activity\cite{15}. The depolarization and calcium dependent synthesis of these compounds, therefore, suggests that the endogenous cannabinoid system plays a compensatory role in dampening seizure activity. Moreover, high concentrations of anandamide are detected in hippocampus an area with high cannabinoid CB$_1$ receptor expression\cite{16}. The hippocampus is known to be a major brain region involved in epileptogenesis and seizure disorders\cite{17}. Thus, endocannabinoids are likely to play an important role in modulating seizure threshold and severity.

Apart from its role in epileptogenesis, anandamide was also found to exert intense emotional and cognitive effects. Cerebrospinal anandamide levels were found to be elevated in acute schizophrenia and were inversely correlated with psychotic symptoms\cite{18}.

Anandamide mediates their effects by binding to cannabinoid CB$_1$ and CB$_2$ receptor\cite{19}. However, it is unlikely that the cannabinoid CB$_2$ receptor mediates the anticonvulsant effect of anandamide because this receptor is not present in brain\cite{20}. Moreover, the CB$_1$ receptor antagonist (AM 281) has been shown to be selective for the cannabinoid CB$_1$ receptor, with negligible binding at cannabinoid CB$_2$\cite{6}. Anandamide can also bind vanilloid receptors 1 (VR1) that are found in the brain\cite{21}. However, it is unlikely that the vanilloid VR1 receptor is anandamide’s anticonvulsant site of action because the selective cannabinoid CB$_1$ receptor antagonist AM 281 completely blocks the anticonvulsant effect of anandamide. Thus, the current data strongly implicate the cannabinoid CB$_1$ receptor as the mechanistic site of action mediating the anticonvulsant effects of endocannabinoids.

Due to the highly lipophilic nature of the majority of endocannabinoids, solubility problems have been encountered during experiments, which have only been overcome by the use of a dispersing vehicle such as Tween 80, ethanol or DMSO. Tween 80 was chosen for the current study following the example of many previous studies\cite{2,3,21}. The anti-convulsant effect of anandamide in the current study was not due to the effect of the drug vehicle Tween 80, which was used to disperse the lipophilic anandamide. Application of Tween 80 at a concentration equivalent to that used to disperse anandamide (10 µM) had no significant effect on either the amplitude of the PS1 or PS2.
The current study provides direct evidence for a physiological role of endocannabinoids in modulating convulsion. In addition, these data further establish the cannabinoid CB₁ receptor and the endogenous cannabinoid system as a potential treatment target for the control of epilepsy. Additional studies investigating the role of this system in epilepsy are clearly warranted.

References


مادة الأناندامايد تقلل من التشنجات الصرعية
في التجربة على الشرائح المخية

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المستخلص. لقد أثبتت مستخلصات الكابانيوبينز المصنعة قدرتها الفائقة على تقليل التشنجات الصرعية سواءً في الدراسات التي تستخدم شرائح مخية أو حيوانات كاملة، وذلك عن طريق تخفيف مستقبلات (س ب 1) العصبية، ولمعرفة إذا كانت مستخلصات الكابانيوبينز المفرزة داخل الجسم لها نفس التأثير فقد استخدمت الدراسة الحالية 24 فأرًا أبيضًا للدراسة تأثير هذه المواد أيضًا على التشنجات الصرعية. وللاختيار تأثير هذه المواد على التشنجات الصرعية، تم استئصال الهمبوكميس من مخ الفأر الأبيض وتم تقطيعه إلى شرائح بسمك 400 ميكرون، وذلك لدراسة تأثيرها في وسائل الكهروفيزيولوجية، والتي تم من خلالها تسجيل الاستجابة العصبية قبل وبعد إضافة الأدوية المختلفة. كما تم قياس طول الاستجابة العصبية والتدوير فيها قبل وبعد إضافة الأدوية المختلفة. وقد تم تحليل النتائج باستخدام برنامج "إي تي بي".

بعد إحداث تشنجات صرعية عن طريق إضافة مادة الباروكوتسين إلى الشريحة المخية، تم إضافة مادة الأناندامايد، والتي قامت بتقليل التشنجات الصرعية بشكل ملموس ومهم إحصائيًا. وعند إضافة مادة "إي إم 281" لم تتمكن مادة الأناندامايد من منع حدوث التشنجات الصرعية. لقد أثبتت الدراسة الحالية قدرة الأناندامايد على تقليل التشنجات الصرعية بشكل مهم وملموس، مما يدل على الدور الكبير وال مهم الذي تلعبه مستقبلات (س ب 1) العصبية في منع حدوث الصرع.