

# Anticonvulsant action of anandamide in an in vitro model of epilepsy

Abdulmonem A. Al-Hayani, DipFMS, PhD.

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## ABSTRACT

**Objectives:** 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 receptors (CB<sub>1</sub>). The current study used 24 adult Sprague Dawley rats to investigate the effects of endogenously occurring cannabinoids (endocannabinoids) on epileptiform activity induced by picrotoxin.

**Methods:** We carried out the study at King Fahad Medical Research Center, Jeddah, Kingdom of Saudi Arabia in September 2004. We made extracellular recordings 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.

**Results:** Using single pulse stimulation, perfusion of 0.5  $\mu$ M 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 CB<sub>1</sub> receptor antagonist, AM281 (500 nM) had no effect on responses recorded in the presence of picrotoxin, but totally blocked the effect of subsequently perfused anandamide.

**Conclusion:** The results showed that anandamide caused an anti-convulsant effect. Furthermore, these results implicate the cannabinoid CB<sub>1</sub> receptor as a major endogenous site of seizure modulation.

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Epilepsy is one of the most common neurological conditions and is characterized by spontaneous recurrent seizures.<sup>1</sup> Understanding the pathophysiology of seizure initiation and termination has important implications for our ability to manage seizure disorders, and for the potential development of novel anti-epileptic agents. Previous research in our laboratory<sup>2</sup> and others<sup>3-5</sup> 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 type 1 receptors

(CB<sub>1</sub>).<sup>2</sup> Two different cannabinoid receptors (CB<sub>1</sub> and CB<sub>2</sub>), have been identified, so far, which were cloned in 1990 and 1993.<sup>6</sup> Cannabinoid receptor CB<sub>1</sub> is the type preferentially expressed in the brain and is known to mediate the psychoactive effect of cannabinoids.<sup>7</sup> The distribution of CB<sub>1</sub> receptors is not homogenous in the brain, CB<sub>1</sub> receptors were found to be very abundant in the hippocampus, neocortical area, and the limbic system, the areas believed to modulate seizure activity.<sup>8</sup> The discovery of cannabinoid receptors was followed in 1992 and 1995 by the demonstration of the existence of endogenous cannabinoid receptor

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From the Department of Anatomy, Faculty of Medicine, King Abdul-Aziz University, Jeddah, Kingdom of Saudi Arabia.

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Address correspondence and reprint request to: Dr. Abdulmonem A. Al-Hayani, Department of Anatomy, Faculty of Medicine, King Abdul-Aziz University, PO Box 80205, Jeddah 21413, Kingdom of Saudi Arabia. Tel. +966 (2) 6400000 Ext. 20121/20122. Fax. +966 (2) 26400000 Ext. 20120. E-mail: hayani30@hotmail.com

agonists.<sup>9</sup> The most important of these are arachidonyl ethanolamide (anandamide) and 2-arachidonylglycerol, where there is evidence that both can serve as neuromodulators or neurotransmitters.<sup>10</sup> As our previous study has shown an anti-epileptic effect of the cannabinoid CB<sub>1</sub> agonist WIN55,212-2,<sup>2</sup> 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.

**Methods.** The study was carried out at King Fahad Medical Research Center, Jeddah, Saudi Arabia in September 2004. Twenty-four young adult Sprague-Dawley rats aged from 5-7 weeks were used in the current study. After general anesthesia with halothane, they were decapitated 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 400µm 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/minute. 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 (PS) 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 PS were then evoked at 30 second intervals until a stable baseline of at least 30 minutes was established. Data were stored and analyzed using the Long Term Potentiation (LTP) program.<sup>11</sup> Drugs were applied by addition to the perfusion medium. Stock solutions of the endocannabinoid CB<sub>1</sub> receptor agonists (anandamide) were made up in alcohol and stored at 4°C. When required they were mixed with Tween 80 (2 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. The AM281 (the cannabinoid CB<sub>1</sub> receptor antagonist) was made up as a 10 mM stock solution in dimethylsulphoxide (DMSO) and diluted in aCSF as required. Epilepsy was induced by using the convulsant poisonous plant derivative 'picrotoxin', which is a non-competitive gamma-aminobutyric acid A receptor antagonist. Picrotoxin has been widely used to induce epilepsy in the in vitro preparations.<sup>12</sup> The response recorded after the application of picrotoxin showed multiple PS which could be named as PS1, PS2, and so forth (**Figure 1**).

In all cases, statistical analysis was performed using the Instant Statistical Package (INSTAT) program to measure significance using the paired student's 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. 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 minutes caused epilepsy by introducing a second PS (PS2) and increasing the amplitude of the first PS (PS1) from the mean baseline value of  $64.3 \pm 5\%$  to  $78.5 \pm 3\%$  (*n*=5).

**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 second PS 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 minutes. Perfusion of anandamide (10 µM) reduced the amplitude of PS1 to  $53.4 \pm 7\%$  of the picrotoxin baseline. The amplitude of PS2 was reduced to  $42.6 \pm 6\%$  of the picrotoxin baseline (*n*=8, **Figure 2**). Anandamide therefore showed a strong anticonvulsant effect.

**Anandamide's anticonvulsant effect is mediated by cannabinoid's CB<sub>1</sub> receptor activation.** A pretreatment dose of the cannabinoid CB<sub>1</sub> receptor antagonist, AM281 (500 nM) was used to test if the effect of anandamide was mediated through the central cannabinoid CB<sub>1</sub> receptors or not. Stable control response was obtained prior to the perfusion of 500 nM picrotoxin. Once a stable second PS was obtained, the CB<sub>1</sub> receptor antagonist AM281 (500 nM) was perfused for 30 minutes. This was followed by a perfusion of anandamide (10 µM) for 30 minutes. The AM281 alone did not affect the amplitude of the PS1 and PS2. After 30 minutes perfusion of AM281, the amplitude of PS1 was  $95 \pm 4\%$  of the picrotoxin baseline (namely, no significant change). The AM281 markedly reduced the effect of the subsequently perfused anandamide (10 µM). The perfusion of anandamide (10 µM) for 30 minutes resulted in a very small reduction of the amplitudes of PS1 and PS2 ( $94 \pm 8\%$  and  $92 \pm 9\%$ ) which were not statistically significant (*n*=6, *p*<0.05, **Figure 3**).

**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 PS. Perfusion of Tween 80 alone for 30 minutes 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 minutes of Tween 80 perfusion, the amplitudes of

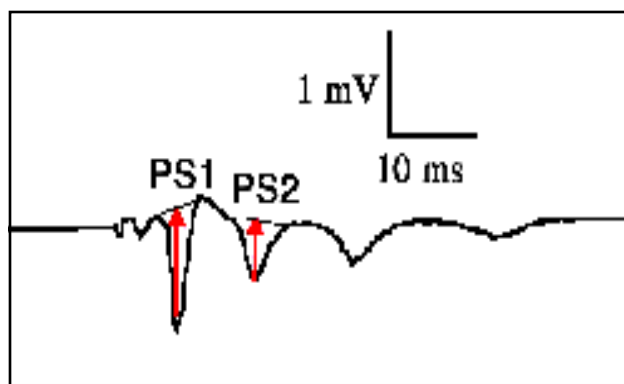


Figure 1 - An example of a synaptic response recorded from the CA1 region of the hippocampal slice showing multiple population spikes (PS) after application of picrotoxin (500 nM). Population spike 1 (PS1) is the first PS and the upward arrow indicates the amplitude (height) of the PS in millivolt (mV). Population spike 2 (PS2) is the second PS and the upward arrow indicates the amplitude (height) of the population spike in mV. Inset shows the time scale of the synaptic response.

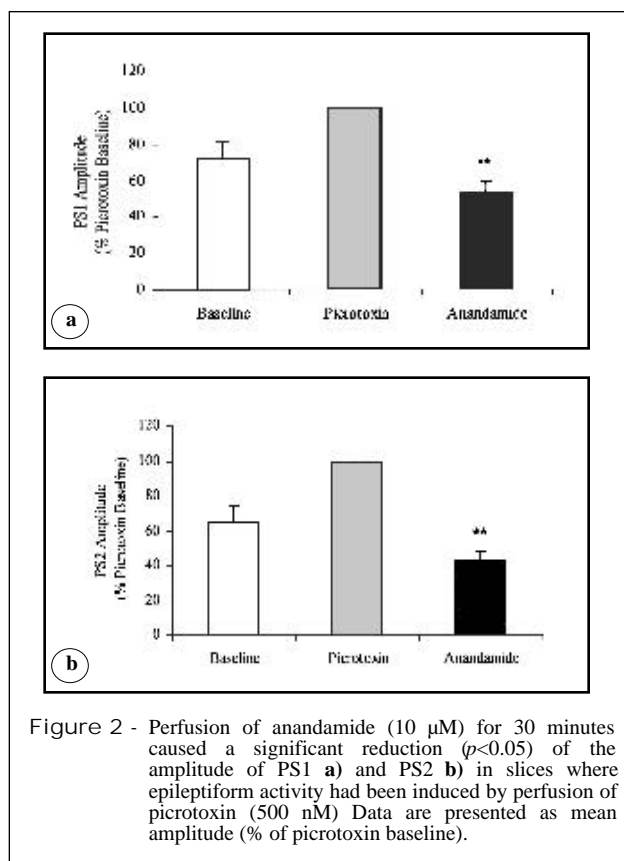


Figure 2 - Perfusion of anandamide (10  $\mu$ M) for 30 minutes caused a significant reduction ( $p < 0.05$ ) of the amplitude of PS1 **a**) and PS2 **b**) in slices where epileptiform activity had been induced by perfusion of picrotoxin (500 nM). Data are presented as mean amplitude (% of picrotoxin baseline).

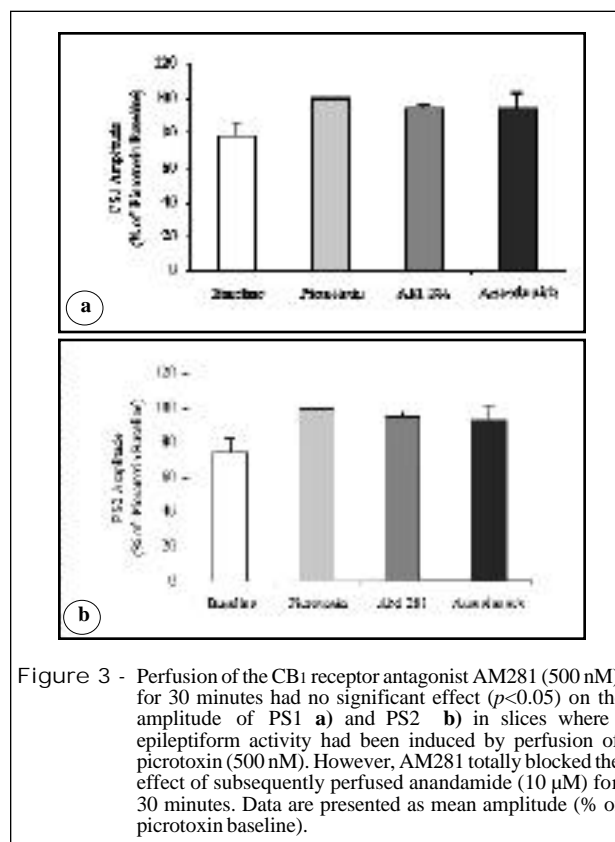


Figure 3 - Perfusion of the CB1 receptor antagonist AM281 (500 nM) for 30 minutes had no significant effect ( $p < 0.05$ ) on the amplitude of PS1 **a**) and PS2 **b**) in slices where epileptiform activity had been induced by perfusion of picrotoxin (500 nM). However, AM281 totally blocked the effect of subsequently perfused anandamide (10  $\mu$ M) for 30 minutes. Data are presented as mean amplitude (% of picrotoxin baseline).

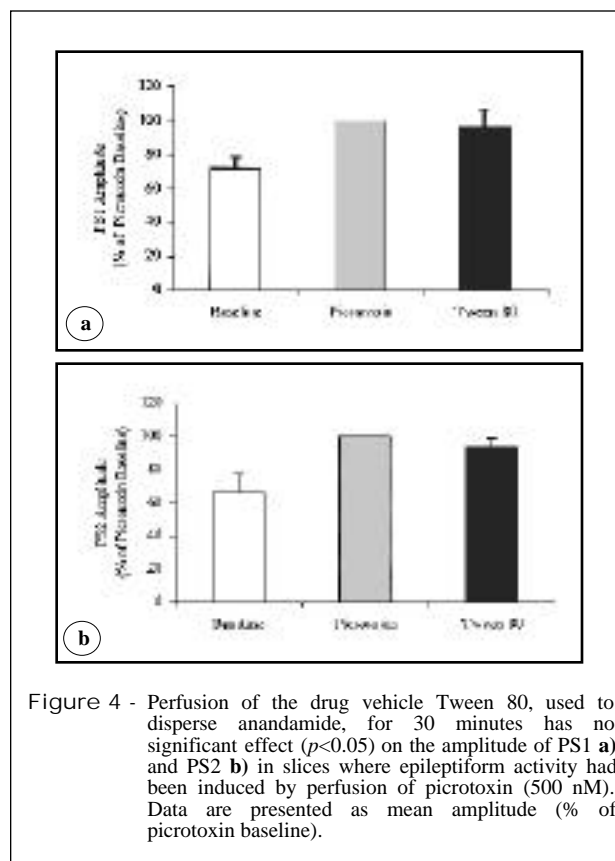


Figure 4 - Perfusion of the drug vehicle Tween 80, used to disperse anandamide, for 30 minutes has no significant effect ( $p < 0.05$ ) on the amplitude of PS1 **a**) and PS2 **b**) in slices where epileptiform activity had been induced by perfusion of picrotoxin (500 nM). Data are presented as mean amplitude (% of picrotoxin baseline).

PS1 and PS2 were  $98 \pm 11\%$  and  $102 \pm 6\%$  of the original baseline. ( $n=5$ ,  $p<0.05$ , **Figure 4**).

**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<sub>1</sub> receptor.

Anandamide was the first putative endogenous ligand for cannabinoid receptors to be identified,<sup>13</sup> and 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 N-arachidonyl-phosphatidylethanolamine. The compound is reported to be synthesized "on-demand" by phospholipase-D in a depolarization and calcium-dependent manner.<sup>14</sup> Previous researchers have shown that elevated intracellular calcium accompanies seizure activity.<sup>15</sup> The depolarization and calcium dependent synthesis of these compounds, therefore, suggest that the endogenous cannabinoid system plays a compensatory role in dampening seizure activity. Moreover, high concentrations of anandamide are detected in the hippocampus, an area with high cannabinoid CB<sub>1</sub> receptor expression.<sup>16</sup> The hippocampus is known to be a major brain region involved in epileptogenesis and seizure disorders.<sup>17</sup> Thus, endocannabinoids are likely to play an important role in modulating seizure threshold and severity.

Anandamide mediates their effects by binding to cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors.<sup>18</sup> However, it is unlikely that the cannabinoid CB<sub>2</sub> receptor mediates the anticonvulsant effect of anandamide because this receptor is not present in the brain.<sup>19</sup> Moreover, the CB<sub>1</sub> receptor antagonist (AM 281) has been shown to be selective for the cannabinoid CB<sub>1</sub> receptor, with negligible binding at cannabinoid CB<sub>2</sub>.<sup>6</sup> Anandamide can also bind vanilloid receptors (VR1) that are found in the brain.<sup>20</sup> However, it is unlikely that the vanilloid VR1 receptor is anandamide's anticonvulsant site of action because the selective cannabinoid CB<sub>1</sub> receptor antagonist AM 281 completely blocks the anticonvulsant effect of anandamide. Thus, the current data strongly implicates the cannabinoid CB<sub>1</sub> receptor as the mechanistic site of action mediating the anti-convulsant 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 dimethylsulphoxide (DMSO). Tween 80 was chosen for the current study following the example of many previous studies.<sup>2,3,20</sup> 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  $\mu$ M) had no significant effect on either the amplitude of 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 CB<sub>1</sub> 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.

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