European Journal of Pharmacology 586 (2008) 160–163

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Short communication

Peripheral cannabinoid CB_1 receptors inhibit evoked responses of nociceptive neurones *in vivo*

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ARTICLE INFO

Article history:
Received 23 November 2007
Received in revised form 20 February 2008
Accepted 3 March 2008
Available online 13 March 2008

Keywords:
Cannabinoid CB_1 receptor
Nociceptor
Antinociception

ABSTRACT

We investigated the effect of peripheral administration of a selective cannabinoid CB_1 receptor agonist arachidonyl-2-choroethylamide (ACEA), on evoked responses of primary afferents *in vivo*. Extracellular recordings were made from filaments of the saphenous nerve that responded to noxious mechanical stimulation of their receptive fields and effects of ACEA (30 and 50 μg/100 μl, i.a.) were studied. ACEA significantly inhibited evoked responses, effects that were blocked by co-administration of the cannabinoid CB_1 receptor antagonist AM251 (30 μg/100 μl). These results demonstrate a cannabinoid CB_1 receptor-mediated inhibition of primary afferent nociceptor excitability and provide further support for a peripheral site of action of cannabinoids.

1. Introduction

Cannabinoids are able to modulate nociception in the periphery (Hohmann, 2002). Evidence exists for both cannabinoid-1 (CB_1) and cannabinoid-2 (CB_2) receptors in peripheral tissues (Maccarrone et al., 2003; Stander et al., 2005). However, little is known about how cannabinoids modulate nociception at this level. It has been postulated that cannabinoid receptor agonists have a direct action on the peripheral terminals of primary afferent neurones (Kelly et al., 2003; Millns et al., 2001; Sagar et al., 2005), but further investigation is required.

Evidence to date suggests an important role for cannabinoid CB_1 receptors. In dorsal root ganglia, cannabinoid CB_1 receptors are expressed by small diameter peptidergic and non-peptidergic neurones as well as large diameter neurones (Ahluwalia et al., 2002; Bridges et al., 2003), where they undergo axonal translocation and insertion on the peripheral endings (Hohmann and Herkenham, 1999). Several previous *in vitro* studies have demonstrated an inhibitory effect of cannabinoid CB_1 receptor activation on primary sensory neurone function (Agarwal et al., 2007; Millns et al., 2001; Sagar et al., 2005) and an inhibition of neuropeptide release (Ellington et al., 2002; Richardson et al., 1998). Peripheral cannabinoid CB_1 receptor activation *in vivo* inhibits spinal somatosensory transmission (Kelly et al., 2003) and is analgesic (Calignano et al., 1998; Gutierrez et al., 2007). Recently it was demonstrated that the analgesic effects of systemically administered cannabinoids are largely abolished in transgenic mice in which the cannabinoid CB_1 receptor is deleted from Nav1.8 expressing C-fibre nociceptors (Agarwal et al., 2007).

Collectively, these aforementioned studies are suggestive of the presence of functional cannabinoid CB_1 receptors on the peripheral terminals of primary afferent nociceptors and imply a critical role in inhibiting their excitability. *In vivo* data on the effect of cannabinoid CB_1 receptor activation on nociceptive neuronal responses is lacking. Further studies are required to fully elucidate the mechanisms of cannabinoid CB_1 receptor mediated peripheral antinociception. Our aim was to test the hypothesis that peripheral cannabinoid CB_1 receptor activation is able to inhibit evoked responses of mechano-nociceptive neurones *in vivo*.

2. Materials and methods

2.1. Animals

Male Wistar rats (B and K, UK) (250–350 g) housed in temperature and humidity controlled holding rooms on a 12:12 h light: dark cycle were used in this study. Food and water were made available *ab libitum*. All experimental procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and IASP guidelines.

2.2. Drugs

Sodium pentobarbital was obtained in its sodium salt form from Sigma (Dorset, U.K) and was made up in house to allow i.p. and i.v. injection for anaesthetic induction and maintenance respectively. Arachidonyl-2-choroethylamide (ACEA) was obtained from Tocris U.K.

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doi:10.1016/j.ejphar.2008.03.003

(Ltd) in 100% ethanol. ACEA was re-suspended in a vehicle of 5% ethanol and saline with tween 80. AM251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophen yl)-4-methyl-1H-pyrazole-3-carboxamide) was also purchased from Tocris and was co-administered with ACEA, or alone in the above vehicle.

2.3. Surgical procedures

Anaesthesia was induced with sodium pentobarbital (60 mg/kg, i.p.) and the external jugular vein and trachea were cannulated, for maintenance of anaesthesia (10 mg/kg/h, i.v.) and clearing of the airways respectively. A cannula for drug delivery was inserted into the right femoral artery and advanced to the point of bifurcation of the descending aorta. The skin and connective tissues overlying the medial aspect of the left leg were incised and the lower part of the leg was attached to a platform for stability. An oil pool was created using skin flaps and filled with warmed (37 °C) mineral oil (Sigma, U.K). The saphenous nerve was dissected free at a point distal to the knee joint and sectioned centrally. The cut saphenous nerve was placed on a dental mirror teased and placed onto bipolar platinum electrodes. The saphenous was teased until fine filaments (typically containing 1–4 afferent fibres) responding to mechanical probing (glass rod with a 1 mm tip) of the dorsal surface of the hindpaw were isolated. The conduction velocities of the units were ascertained by electrical stimulation the receptive field (0.3 Hz, 0.5 ms pulse duration, 3–7 mA).

A mechanical stimulator (Dept Physiology, Mechanical workshop) was positioned over the most responsive area of the receptive field and noxious mechanical stimuli (~100 g, 5 s duration @ 5 min intervals) were delivered and evoked activity recorded. Evoked responses were recorded until stable and the mean afferent firing rate associated with the latter 2–3 stimuli were taken as control baseline level which was normalised to 100%. Neuronal activity was digitised by a data acquisition system (CED1401, Cambridge Electronic Design, Cambridge, UK) and stored on a PC for off-line analysis. The mean firing rate/mechanical stimulus was determined using Spike 2 software (Cambridge Electronic Design, Cambridge, UK) and the % of control response was calculated.

2.4. Drug administration

Effects of 100 μl vehicle (5% ethanol in saline and tween 80) (n=6) or ACEA (30 and 50 μg) (Tocris, U.K) (n=7/6) on mechanically-evoked responses were followed for 40 min at 5 minute intervals. In separate groups of rats the effect of ACEA in the presence of AM251 (30 μg/100 μl) (n=7) and the effects of AM251 (30 μg/100 μl) alone (n=7) on noxious mechanically-evoked firing of primary afferents were studied. All drugs were injected by close intra-arterial injection.

2.5. Statistics

Data are expressed as mean and s.e.m. Statistical analysis was carried out with unpaired t-test and one way ANOVA, where appropriate, $^{*}P<0.05$, $^{**}P<0.01$.

3. Results

3.1. Responses of recorded units to noxious mechanical stimulation

Multi-unit recordings were made from primary afferent neurones of the saphenous nerve that innervate the dorsal surface of the rat hindpaw. In each experiment, recordings were made from 1–4 mechanosensitive afferents. Conduction velocities ranged from 10.38–0.29 ms^{-1}, placing them in the Aδ- and C-fibre velocity range (Lynn and Carpenter, 1982). All the units recorded responded to mechanical stimulation of the receptive field with a glass rod (but not brush) and exhibited slowly adapting responses to 5 s noxious mechanical stimulation of the receptive field.

3.2. Effect of ACEA on the responses of primary afferent neurones to noxious mechanical stimulation

Peripherally administered ACEA (30 and 50 μg/100 μl, i.a.) inhibited noxious mechanically-evoked responses of primary afferent neurones (Figs. 1 and 2). The inhibitory effect of ACEA (30 and 50 μg) was significant compared to that of vehicle (P<0.01 and P<0.001 respectively) (Fig. 1). Furthermore, the mean maximal inhibitory effect of ACEA 30 and 50 μg was greater than that of vehicle (Fig. 2A). Area under the curve analysis (AUC) over the full time course (0–40 min) demonstrated that ACEA (30 and 50 μg/100 μl, i.a.) had significantly greater inhibitory effects compared to vehicle (Fig. 2B).

To confirm the involvement of peripheral cannabinoid CB_1 receptors in mediating the inhibitory effects of ACEA, co-administration of the cannabinoid CB_1 receptor antagonist AM251 was carried out. The inhibitory effects of ACEA (50 μg/100 μl) were completely blocked by the co-administration of AM251 (30 μg/100 μl, i.a.) (Figs. 1B and 2) and the mean maximal inhibitory effect of ACEA in the presence of AM251 was not significantly different to that of vehicle (Fig. 2A). The AUC of the effect of ACEA in the presence of AM251 was significantly greater than ACEA alone (P<0.05) (Fig. 2B). These data indicate that the cannabinoid CB_1 receptor is solely responsible for mediating the reported antinociceptive effects of ACEA. In our hands, AM251 had only minor effects when administered alone, the time course of effect of AM251 was

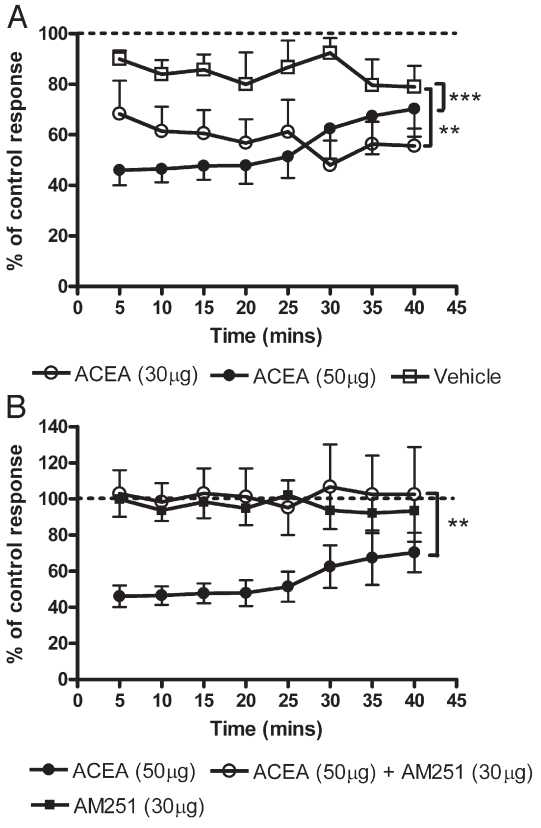

Fig. 1. ACEA inhibits noxious mechanically-evoked responses of primary afferent neurones via CB_1 receptor activation. (A) ACEA (30 and 50 μg/100 μl, i.a.) (n=7/6) had a dose dependent inhibitory effect on mechanically-evoked responses. Vehicle (5% ethanol/saline/tween 80) (n=6) had minor effects on mechanically evoked responses. (B) The inhibitory effect of ACEA (50 μg/100 μl, i.a.) was blocked by the co-administration of AM251 (30 μg/100 μl, i.a.) (n=7). AM251 administration alone (n=7) had only minor effects on mechanically-evoked responses. Data analysed with Kruskall Wallis one-way ANOVA, $^{**}P$<0.01, $^{***}P$<0.001 vs vehicle.

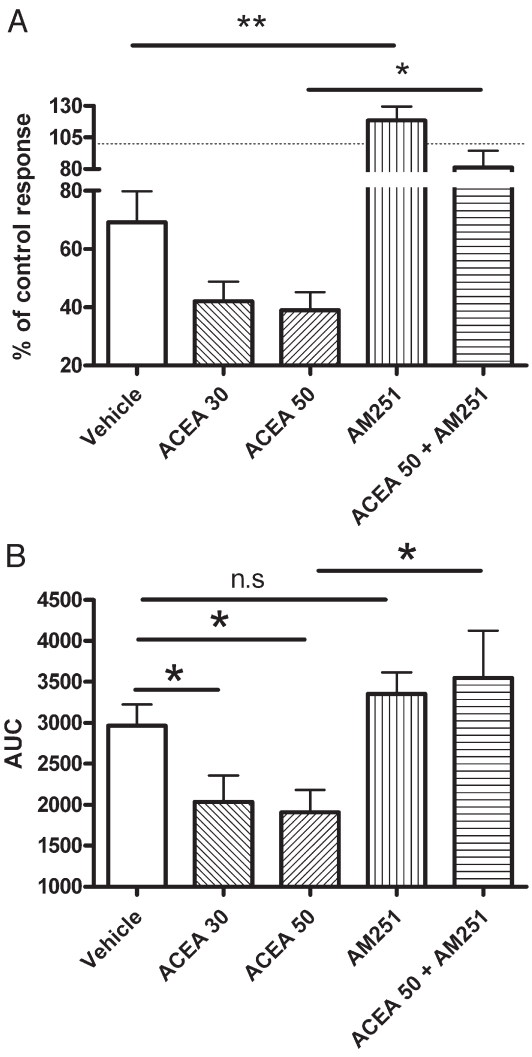

Fig. 2. Analysis of the inhibitory effects of ACEA on noxious mechanically-evoked responses of primary afferent neurones. (A) The mean maximal inhibitory effect of ACEA (30 and 50 μg/100 μl, i.a.) (n=7/6) was greater than that of vehicle (5% ethanol/saline/tween 80) (n=6) and ACEA (50 μg/100 μl, i.a.) when in combination with AM251 (30 μg/100 μl, i.a.) (n=7). The mean maximal facilitatory effect of AM251 (30 μg/100 μl, i.a.) (n=7) was significant compared to vehicle. (B) Area under the curve (AUC) analysis for the effect of vehicle (n=6), ACEA alone (n=7/6) and when co-administered with AM251 (n=7), and the effect of AM251 alone (n=7). Data analysed with Mann Whitney, $^{*}P<0.05$, $^{**}P<0.01$.

not significantly different to that of vehicle (Fig. 1B). A small but significant facilitatory effect was seen when the mean maximal effects of AM251 and vehicle were compared ($P<0.01$) (Fig. 2A), however the AUC of the effect of AM251 and vehicle were similar (Fig. 2B).

4. Discussion

The anatomical locus and receptor mechanisms that underlie the peripheral analgesic effects of cannabinoids are unknown. Our data is the first *in vivo* electrophysiological evidence suggesting that activation of cannabinoid CB_1 receptors on the peripheral terminals of nociceptive neurones inhibits their responsiveness to noxious stimulation. Here, selective activation of cannabinoid CB_1 receptors by ACEA was able to inhibit mechanonociceptive (both Aδ- and C-fibre) neuronal responses, effects that were blocked by the cannabinoid CB_1 receptor antagonist AM251. ACEA had a rapid onset of effect (inhibition by the time the first stimulus was delivered) and the saphenous nerve was sectioned proximally removing the possibility of central modulation. We used a local intra-arterial injection method and low concentrations of ACEA that are unlikely to have systemic effects (Hillard et al., 1999; Kelly et al., 2003). For these reasons we are confident that the effects of ACEA reported here were mediated at the peripheral level and our data suggests a direct effect on the primary afferent terminal. The existence of a peripheral endocannabinoid tone is controversial (Calignano et al., 1998; Kelly et al., 2003; Richardson et al., 1998). In the present study we saw only a minor facilitatory effect of AM251 on mechanically evoked responses at concentrations used previously that attenuated the antihyperalgesic effects of WIN 55,212-2 (Johanek and Simone, 2004). Therefore the present data cannot distinguish between the presence of an endocannabinoid tone and an inverse agonist effect of AM251.

High concentrations of ACEA have TRPV1 mediated effects on sensory neurones (Baker and McDougall, 2004). However, this is unlikely under our experimental conditions as the concentrations used are low and the inhibitory effects of ACEA are completely abolished by AM251, suggesting a cannabinoid CB_1 receptor specific action. Furthermore, the potent and selective TRPV1 antagonist iodoresiniferatoxin has no effect on the peripheral antinociceptive effects of ACEA (Kelly et al., 2003).

The present study and others implicate an important role for the cannabinoid CB_1 receptor in the modulation of nociceptor excitability. It is possible that this effect is mediated by the modulation of ion channel function (Binzen et al., 2006; Sade et al., 2006). Cannabinoid CB_1 receptors are localised appropriately to contribute to the aforementioned effects as they are expressed by both A- and C-fibres (as studied here) and co-expressed with markers of nociceptive neurones (Agarwal et al., 2007; Ahluwalia et al., 2002; Hohmann and Herkenham, 1999). Activation of peripheral cannabinoid CB_1 receptors inhibits neuropeptide release (Ellington et al., 2002; Richardson et al., 1998) and suppresses c-Fos protein like immunoreactivity, in areas of the dorsal horn associated with the termination sites of nociceptors (Nackley et al., 2003). In rodent models of pain, the antinociceptive effects of peripherally administered cannabinoid agonists were reversed by cannabinoid CB_1 receptor antagonists (Johanek and Simone, 2004; Nackley et al., 2003; Richardson et al., 1998). What's more, selective activation of peripheral cannabinoid CB_1 receptors by ACEA inhibited noxious mechanically-evoked responses of spinal neurons (Kelly et al., 2003). In each of these studies it was hypothesised that effects were mediated by activation of cannabinoid CB_1 receptors on the peripheral terminals of primary afferent neurones. The findings of the present study and the previous demonstration of effects of ACEA on dorsal root ganglia neurones in culture (Sagar et al., 2005) strengthen this hypothesis.

CNS side effects, such as anxiety and sedation and also abuse potential have limited the re-introduction of cannabinoids as medications. The present study and evidence from earlier studies, suggests that cannabinoid CB_1 receptors located on neurones outside the CNS can mediate potent antinociceptive effects and may be an important target for cannabinoid derived analgesics. The findings of this study augment our understanding of the mechanisms of cannabinoid induced analgesia.

Acknowledgements

This study was supported by the Wellcome Trust U.K. (Ref 066759).

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