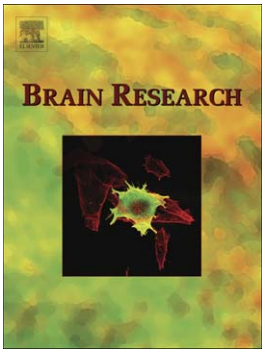

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AM404 decreases Fos-immunoreactivity in the spinal cord in a model of inflammatory pain.

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Abstract

Cannabinoids, such as anandamide are involved in pain transmission. We evaluated the effects of AM404 (N-(4-hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide), an anandamide reuptake inhibitor, monitoring the expression of c-fos, a marker of activated neurons and the pain-related behaviours using formalin test. The study was carried out in an experimental model of inflammatory pain made by a single injection of formalin in rat hind paws. Formalin test showed that the antinociceptive effect of AM404 was evident in phase I. We found that Fos-positive neurons in dorsal superficial and deep laminae of the lumbar spinal cord increased in formalin injected animals and that AM404 significantly reduced Fos induction. Co-administration of cannabinoid CB_1 receptor antagonist (AM251), cannabinoid CB_2 receptor antagonist (AM630) and transient receptor potential vanilloid type 1 (TRPV-1) antagonist (capsazepine), attenuate the inhibitory effect of AM404 and this effect was higher using cannabinoid CB_2 and vanilloid TRPV-1 receptor antagonists. These results suggest that AM404 could be a useful drug to reduce inflammatory pain in our experimental model and that cannabinoid CB_2 receptor and vanilloid TRPV-1 receptor, and to a lesser extent, the cannabinoid CB_1 receptor are involved.

Section: Sensory and Motor Systems

Keywords: inflammatory pain, anandamide, cannabinoid receptors, TRPV-1 receptor, spinal cord, Fos.

Abbreviations:
AM404, N-(4-hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide
AM251, (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide)
AM630, (6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl](4-methoxyphenyl)
capsazepine, (N-[2-(4-Chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide)
DMSO, dimethylsulphoxide
TRPV-1, transient receptor potential vanilloid type 1

1. Introduction

Inflammation is now recognized as an overwhelming burden to the healthcare status of our population and the underlying basis of a significant number of diseases. The elderly generally bear the burden of morbidity and mortality, which may be reflective of elevated markers of inflammation resulting from decades of lifestyle choices.

Intense noxious stimuli and tissue inflammation produce pain hypersensitivity that is caused by both peripheral sensitization and central sensitization (Woolf and Salter, 2000). The endocannabinoids, which include anandamide (arachidonylethanolamide) and 2-arachidonylglycerol, are a class of lipid compounds that are produced in the brain and other tissues through stimulus-dependent cleavage of membrane lipid precursors (Piomelli et al., 2000) suggesting the presence of a specific membrane transporter (Di Marzo and Deutsch, 1998).

Anandamide binds the cannabinoid CB_1 receptor and causes agonist activity (Di Marzo and Deutsch, 1998). Nevertheless some recent data suggest that anandamide also activates cannabinoid CB_2 receptor (Pertwee and Ross, 2002) and transient receptor potential vanilloid type 1 (TRPV-1), co-expressed with cannabinoid CB_1 receptor (Ross, 2003; Zygmunt et al., 2000). A number of reports have highlighted the anti-inflammatory activity of peripheral cannabinoid CB_1 receptor agonists, indeed intraplantar application of the selective cannabinoid CB_1 receptor antagonist SR141716A in mice was found to enhance pain behaviours elicited by formalin injection (Calignano et al., 1998). Richardson et al. (1998) also showed that peripheral anandamide inhibited hyperalgesia induction after carrageenan injection into rat paws. In order to further clarify the cannabinoid CB_1 and cannabinoid CB_2 receptors role in a model of carrageenan-inflammatory pain, Clayton et al. (2002) demonstrated that both cannabinoid CB_1 and cannabinoid CB_2 receptors are implicated in inflammatory hypersensitivity. Cannabinoid CB_2 receptor in the periphery suppresses the

development of inflammatory pain as well as inflammation-evoked neuronal activity in the central nervous system (Nackley et al., 2003; Quartilho et al., 2003).

The vanilloid TRPV-1 receptor responds to noxious stimuli including capsaicin, heat and extracellular acidification, and it is able to integrate simultaneous exposure to these stimuli (Caterina et al., 1997; Tominaga et al., 1998). The findings linking capsaicin with nociceptive behaviour (Walker et al., 2003) led to consider vanilloid TRPV-1 receptor important for pain sensation. Local application of vanilloid TRPV-1 receptor antagonist, capsazepine, and other antagonists was shown to produce a marked antinociception in formalin model of pain in mice, and to reduce inflammation-induced thermal hyperalgesic responses in the carrageenan model of pain in rats (Kwak et al., 1998; Santos and Calixto, 1997).

Recently an anandamide transport inhibitor has been developed, i.e. the drug N-(4-hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (AM404). AM404 is an anandamide re-uptake inhibitor which enhances the antinociceptive and the hypotensive effects of anandamide *in vivo* (Beltramo et al., 1997; Calignano et al., 1997). Moreover we have showed that this drug is useful to reduce Fos expression in the spinal cord in a model of neuropathic pain (Rodella et al., 2005).

On the basis of these findings, the aim of this study was to evaluate the effects of the administration of AM404 and the involvement of cannabinoid CB_1, cannabinoid CB2 and vanilloid TRPV-1 receptors in the spinal cord in a localized inflammatory pain model using behavioural formalin test and c-fos expression, a marker of activated neurons (Coggeshall, 2005; Hunt et al., 1987).

2. Results

First experimental protocol

In the NAIVE groups the number of Fos positive neurons in laminae I-II and III-VI was very low without any significant difference between the two different groups of laminae. Fos distribution pattern found in the vehicle injected animals and in AM404 injected animals, without formalin injection, was similar to the NAIVE. In the i.p. vehicle injected animals followed by formalin we observed a significant increase of Fos positive neurons scattered in the laminae I-II and in laminae III-VI compared with the NAIVE, vehicle and AM404 groups. The data on the distribution of the labelled cells in the spinal cord in the various laminae are represented in Fig. 1.

Second experimental protocol

In the formalin injected/vehicle treated animals, numerous Fos-positive neurons were scattered mainly in the laminae III-VI, but also in I-II.

In the animals injected with formalin and treated with AM404, we observed a significant decrease of Fos positive neurons mainly in the laminae I-II.

In the animals injected with formalin followed by AM251 or AM630 we did not observe a significant increase of Fos positive neurons respect to the animals injected only with formalin.

In the animals injected with formalin followed by capsazepine we did not observe a significant decrease of Fos positive neurons respect to the animals injected only with formalin.

In the animals that received formalin injection followed by the administration of AM251 and AM404, we observed a significant decrease of Fos positive neurons in laminae I-II and III-VI compared to the animals formalin injected and vehicle treated only. In the rats that received formalin injection followed by the administration of AM404 and AM630 or capsazepine, we did not observe a significant decrease of Fos positive neurons in the spinal cord respect to the animals injected with formalin.

The data on the distribution of the labelled cells in the different laminae of spinal cord are represented in Fig. 2 and 3.

The formalin test showed a typical flinching behaviour. Flinching behaviour was biphasic. The first phase started immediately after formalin injection, decreasing gradually in about 10 min. The second phase started at 15 min and latest until 60 min. Systemic administration of AM404 in rat produced a dose-dependent reduction that was significant in phase I but not in phase II nociceptive behaviour (Fig.4). AM251, AM630 and capsazepine was unable to reduce flinching behaviour both in phase I and in phase II. The co-treatment with AM404 and AM251 caused a significant reduction of flinching behaviour. The co-treatment with AM404 and AM630 or capsazepine did not cause a significant reduction of flinching behaviour both in phase I and in phase II.

3. Discussion

The superficial dorsal horn of the spinal cord, particularly the substantia gelatinosa (lamina II of Rexed), is thought to be the target of drugs which modulate nociceptive transmission through primary-afferent Ad- and C-fibres from the periphery (Trettel and Levine, 2003). Among these drugs, anandamide and capsaicin modulate pain transmission in this spinal area through the activation of cannabinoid CB_1 receptors and vanilloid TRPV-1 receptors, respectively (Luo et al., 2002; Morisset et al., 2001; Yang et al., 1998, 1999, 2000). The existence of anandamide transporter is based on an anandamide uptake process that is temperature-dependent, selective and saturable (Beltramo et al., 1997; Ligresti et al., 2004; Moore et al., 2005). In addition several studies identified compounds that inhibit anandamide accumulation into the cells with potential therapeutic value, including AM404 (Beltramo et al., 1997; Costa et al., 2006; Morisset et al., 2001, Rodella et al., 2005;). However, some Authors suggested that anandamide uptake was a process of simple diffusion and that AM404

was actually inhibitor of FAAH (Day et al., 2001; Deutsch et al., 2001; Glaser et al., 2003; Kaczocha et al., 2006).

In this study we evaluated the effects of AM404, a drug interacting in the anandamide pathway by its reuptake inhibition (Beltramo et al., 1997; Calignano et al., 1997; Rodella et al., 2005), at lumbar spinal cord level in an experimental model of inflammatory pain. The animals injected with formalin followed by AM404 administration showed a decrease in the number of Fos positive neurons in the dorsal horn of spinal cord supporting recent data showing the antinociceptive effects of AM404 in a formalin mouse model (La Rana et al., 2006). The involvement of superficial (laminae I-II) and deep laminae (laminae III-VI) (Abbadie et al., 1997) could indicate the involvement of different nociceptive pathways. In addition monitoring the animal behaviour we observed a significant antinociceptive effect of AM404 only in phase I, whereas phase II appear uneffected. This could be due by different messengers involved in the second formalin phase (Potes et al., 2006) or by a fast degradation of AM404 (Fegley et al., 2004; La Rana et al., 2006). We can hypothesize that the Fos data could be representative of phase I formalin pain behaviour because in our study Fos was monitored at 90 min after formalin injection, that is the useful time to evaluate Fos protein expression. Moreover, the correlation between Fos and behaviour is not always direct and may be uncoupled under certain circumstances (Gilron et al., 1999; Hammond et al., 1998).

Our results showed that the cannabinoid CB_1, cannabinoid CB_2 and vanilloid TRPV-1 receptors were differently involved in the pain modulation suppressing selectively their analgesic action with the use of their proper antagonists.

About the involvement of cannabinoid CB_1 receptor, we observed a significant decrease of Fos positive neurons and in flinch number in phase I formalin test in the animals pre-treated with AM251 and AM404. These observations are consistent with previous studies (Calignano et al., 1998; Richardson et al., 1998) showing the anti-inflammatory effect of peripheral

cannabinoid CB_1 receptor agonists. In addition our data are consistent with the data of Martin et al. (1999) showing an increase of Fos expression in lumbar spinal cord in an inflammatory pain model after administration of a cannabinoid CB_1 receptor antagonist.

About the involvement of cannabinoid CB_2 receptor, we did not observe a significant decrease of Fos positive neurons and in flinch number in formalin test in the animals pre-treated with the cannabinoid CB_2 receptor antagonist AM630 and AM404. These data corroborate many studies that have highlighted the anti-nociceptive and anti-hypersensitivity effect in nociceptive and inflammatory pain of cannabinoid CB_2 receptor agonists (Clayton et al., 2002).

So, our data underline the implication of cannabinoid CB_1 and cannabinoid CB_2 receptors in inflammatory hypersensitivity and that the cannabinoid CB_2 receptors are the most involved (Clayton et al., 2002; Nackley et al., 2004; Quartilho et al., 2003). Nevertheless, in a mouse model of inflammatory pain La Rana et al. (2006) reported the pivotal role of cannabinoid CB_1 receptor. This could be due to the species-specificity of cannabinoid receptors / fatty acid amide hydrolase distribution (Egertova et al., 2003; Harkany et al., 2003) and aminoacids sequences (Brown et al., 2002).

Finally, we analyzed the involvement of the vanilloid TRPV-1 receptor using capsazepine. We did not observe any significant changes on Fos expression or behaviour after formalin injection in the animals treated with capsazepine alone or with AM404. Capsazepine is usually ascribed among the vanilloid TRPV-1 antagonists and it has been shown to competitively inhibit capsaicin-mediated responses in several experimental model using different animal species: rat (Bevan et al., 1992a,b), mouse (Santos and Calixto, 1997; Urban and Dray, 1991), and guinea pig (Fox et al., 1995). In addition, it is important considering that some studies indicate possible species' differences in the pharmacology of the vanilloid receptor respect to a specific kind of pain. Walker et al. (2003) observed that the capsazepine did not affect

mechanical hyperalgesia in the Freund's complete adjuvant-inflamed hind paw of the rat or mouse. Moreover, it also produced significant reversal of carageenan-induced thermal hyperalgesia in the guinea pig but was ineffective in the rat.

Our results can be explained by the specie-specificity of capsazepine, which have a low power as antagonist in a rat model of inflammatory pain (Kwak et al., 1998) and by the ability of AM404 to be a full agonist of the vanilloid TRPV-1 receptor with similar kinetics of capsaicin and anandamide (Jennings et al., 2003; Smart and Jerman, 2000; Zygmunt et al., 2000). So, AM404 can interact with both the anandamide transporter (AMT) and vanilloid TRPV-1 receptor (De Petrocellis et al., 2000; Ralevic et al., 2001; Ross et al., 2001; Zygmunt et al., 2000). Some studies (Costa et al., 2006; Palazzo et al., 2006) support these hypothesis suggesting that both vanilloid TRPV-1 and cannabinoid receptors are involved in AM404 modulation of neuropathic pain.

In conclusion, our results agree with findings pointing out that AM404 decreases neuronal activation inhibiting the re-uptake of anandamide (Calignano et al., 1997) in a formalin model of inflammatory pain. Moreover, we demonstrated that the administration of cannabinoid CB_1, cannabinoid CB_2 and vanilloid TRPV-1 receptor antagonists significantly attenuate the inhibitory effect of AM404 in a formalin rat model of inflammatory pain.

Further studies will be performed particularly paying attention to vanilloid TRPV-1 which seems to play a new important role in the analgesic therapeutic strategies.

4. Experimental procedure

4.1 Animal maintenance and preparation

Experiments were carried out on 60 male Sprague-Dawley rats (200 gr. b.wt.). To minimize the circadian variations, the animals were housed in individual cages with food and water *ad libitum* and kept in an animal house at a constant temperature of 22°C with 12 h alternating light-dark cycles. The experiments were performed between 08:00 h and 12:00 h. All effort was made to minimize animal suffering and the number of animals used. The experimental procedures were approved by the Italian Ministry of Health and followed the guidelines for the treatment of animals of the International Association of the Study of Pain (Zimmermann, 1983).

4.2 Compound supply and preparation

Neutral formalin (Sigma, St. Louis, USA) was prepared in a phosphate buffer to give a final concentration of 5%.

The drugs used during the experiments were: AM404 (N-(4-hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide), an inhibitor of anandamide reuptake; AM251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide), a selective cannabinoid CB_1 receptor antagonist (Akerman et al., 2004; Dogrul et al., 2003); AM630 (6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl](4-methoxyphenyl), a cannabinoid CB_2 receptor antagonist (Hosohata et al., 1997) and capsazepine (N-[2-(4-Chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide), a vanilloid TRPV-1 receptor antagonist (Harrison et al., 2003). All the drugs were diluted in a physiological saline containing 10% DMSO (dimethylsulphoxide; Sigma, St. Louis, USA), and purchased from Tocris Cookson Ltd, Bristol, UK.

4.3 Experimental groups

The animals were subdivided into two main experimental groups: the first experimental protocol was performed to test the effects of formalin or AM404 alone monitoring the Fos-induction; the second experimental group was performed to test the effect of the administration of AM404 with the other drugs.

4.3.1 First experimental protocol

The animals (*n*=20) were subdivided into 4 groups: 1) NAÏVE animals (*n*=5), the animals were not treated; 2) vehicle control animals (*n*=5), the animals were injected i.p.(intraperitoneally) with the drug vehicle and after 60 min s.c.(subcutaneously) in the hind paw with the formalin vehicle; 3) AM404 control animals (*n*=5), the animals were injected i.p. with AM404 (10 mg/kg) and after 60 min injected in the hind paw with the vehicle of formalin; 4) formalin control animals (*n*=5), the animals were injected i.p. with the vehicle of AM404 and after 60 min injected subcutaneously in the plantar surface of the left hind paw with neutral formalin (50 ul).

4.3.2 Second experimental protocol (formalin group)

All animals (*n*=40) were subdivided into 8 pharmacological groups: 1) formalin control animals (*n*=5), the animals received the drug vehicle and after 1h were injected with formalin as in the first experimental protocol; 2) AM404 animals (*n*=5), the animals received AM404 (10 mg/Kg) and after 1h they were injected with formalin; 3) AM251 animals (*n*=5), the animals received AM251 (1 mg/Kg) and after 90 min they were injected with formalin; 4) AM630 animals (*n*=5), the animals received AM630 (1 mg/Kg) and after 90 min they were injected with formalin; 5) capsazepine animals (*n*=5), the animals received capsazepine (1 mg/Kg) and after 90 min they were injected with formalin; 6) AM404 and AM251 co-treated animals (*n*=5), the animals received AM251 (1 mg/kg), after 30 min they received AM404 (10 mg/kg) and after 60 min they were injected with formalin; 7) AM404 and AM630 co-treated

animals (*n*=5), the animals received AM630 (1 mg/kg), after 30 min they received AM404 (10 mg/kg) and after 60 min they were injected with formalin; 8) AM404 and capsazepine co-treated animals (*n*=5), the animals received capsazepine (1 mg/kg) 30 min before AM404 (10 mg/kg) and and after 60 min formalin injected. The drug doses corresponded to those used in previous studies (Del Arco et al., 2002; Giuffrida et al., 2000; La Rana et al., 2006; Rodella et al., 2005). Moreover, in our preliminary study we tested the dose response of AM404 in a range of 1-20 mg/Kg and we found that, according to La Rana et. al (2006), the dose of 10 mg/Kg was efficient for acute treatment.

Formalin test Pain and antinociception were assessed by the formalin test, according to Ortiz et al. (2003). Rats were placed in open Plexiglas observation chambers for 30 min to allow them to accommodate to their surroundings, then they were removed for formalin administration. 50 ul of diluted formalin (5%) was injected subcutaneously into the plantar surface of the left hind paw with a 30-gauge needle. Animals were then returned to the chambers, and nociceptive behaviour was observed immediately after formalin injection. Mirrors were placed to enable unhindered observation. Nociceptive behaviour was quantified as the number of flinches of the injected paw during 1-min periods every 5 for 60 min after injection (Malmberg and Yaksh, 1992; Wheeler-Aceto and Cowan, 1991). Flinching was readily discriminated and was characterized as rapid and brief withdrawal or flexing of the injected paw. Formalin-induced flinching behaviour is biphasic. The initial acute phase (0-10 min) is followed by a relatively short quiescent period, which is then followed by a prolonged tonic response (15-60 min). All observations in both phases of the formalin test were carried out by two unaware investigators of the animal group assignment. Results are presented as mean±SEM. Time-courses of antinociceptive response of individual drugs and the combination were constructed by plotting the mean number of flinches as a function of time.

The area under the number of flinches against time curves (AUC) was calculated by the trapezoidal rule.

Fos immunohistochemistry After 90 min from the formalin injection the animals were anaesthetised with sodium pentobarbitone (40 mg/kg i.p.) and transcardically perfused with saline followed by 1L of 4% paraformaldehyde in phosphate buffer 0.1 M pH 7.4. After fixation, the lumbar spinal cord (L4-L6) of each animal was removed, post-fixed in 4% paraformaldehyde in phosphate buffer for 2 h and cryoprotected overnight in 30% sucrose at 4°C. We identified the lumbar spinal cord segments by the presence of lumbar enlargement. The lumbar L4-L6 segments from each rat was determined on the spinal cord in situ using a dissecting microscope and measuring the distance between the points of entry of the most rostral and most caudal rootlets of the L4, L5 and L6 dorsal root. Frozen serial transverse sections (25 μm thick) of all segments were collected in phosphate-buffered saline. We collected all the sections of the L4-L6 segments. For Fos immunohistochemistry, we processed a total of 25 sections per animal. In addition, some sections were Nissl-stained for morphological control. Briefly, the first series of sections were incubated in normal goat serum (10% in phosphate-buffered saline containing 0.1% Triton X-100) for 30 min and then incubated in rabbit polyclonal primary antiserum directed against Fos (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 in phosphate-buffered saline containing 3% normal goat serum and 0.1% Triton X-100, for 24 h at 4°C. After incubation in the primary antiserum, the sections were sequentially incubated in biotinylated goat anti-rabbit immunoglobulins (IgG) and avidin-biotin peroxidase complex (Vector Labs., Burlingame, CA, USA). The reaction product was visualized using hydrogen peroxide and diaminobenzidine (DAB) (Sigma, St. Louis, MO, USA) as chromogen. The immunohistochemistry control was performed by omitting the primary antibody and incubating the sections with non-immune rabbit serum. The distribution of the labelled cells of all animals was charted using an image

analyzer (Immagini e Computer, Milano, Italy). The density of the labelled cells in the lumbar spinal laminae, conventionally grouped into two groups (I-II and III-VI), was evaluated using a quantitative method by researchers unaware of the animal group assignment. Cell counts were made in all processed sections at a final X 40 magnification. The number of Fos-positive cells of each section of each spinal cord was divided by the number of counted tissue sections, in order to evaluate the average number of labelled cells for each animal. The data from all animals were analyzed and compared by analysis of variance (ANOVA) and by a Bonferroni multiple comparison test.

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Figure legends

Fig. 1: Quantitative evaluation of Fos immunoreactivity in L4-L6 spinal cord laminae I-II (a) and III-VI (b) in NAIVE rats, vehicle injected rats, AM404 treated rats and formalin injected rats. Values are mean and show the number of neurons per section ± S.D.; **P*<0.05 compared to NAIVE.

Fig.2: Quantitative evaluation of Fos immunoreactivity in L4-L6 spinal cord laminae I-II (a) and III-VI (b) in the formalin injected rats treated with different drugs. Vehicle: animals injected with the drug vehicle; AM404: animals injected with AM404; AM404 plus AM251: animals injected with AM251 and AM404; AM404 plus AM630: animals injected with AM630 and AM404; AM404 plus CPZ: animals injected with capsazepine and AM404; CPZ: animals injected with capsazepine. Values are mean and show the number of neurons per section ± S.D.; **P*<0.05 compared to vehicle, °*P*<0.05 compared to AM404.

Fig. 3: Fotomicrographs of Fos-positive neurons in the dorsal horn of spinal cord (L5) of formalin injected animals: a) vehicle treated animals; b) AM404 treated animals; c) AM404 plus AM630 treated animals. Arrows indicate Fos-positive neurons. Bar 90 μm.

Fig. 4: Phase I (a) and phase II (b) of the formalin test. Vehicle: animals injected with the drug vehicle; AM404: animals injected with AM404; AM251: animals injected with AM251; AM630: animals injected with AM630; CPZ: animals injected with capsazepine; AM404 plus AM251: animals injected with AM251 and AM404; AM404 plus AM630: animals injected with AM630

and AM404; AM404 plus CPZ: animals injected with capsazepine and AM404. Data are expressed as the area under the number of flinches against time curve (AUC). Values are mean ± SEM; **P*<0.05 compared to vehicle, °*P*<0.05 compared to AM404.