Clinical and Experimental Pharmacology and Physiology (2010) **37**, 544–550 doi: 10.1111/j.1440-1681.2010.05346.x

Peripheral antinociceptive effect of 2-arachidonoyl-glycerol and its interaction with endomorphin-1 in arthritic rat ankle joints

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SUMMARY

1. Both cannabinoid and opioid receptors are localized at the peripheral level, and drugs acting on these receptors may produce antinociception after topical administration; however, the effect of endogenous ligands at these receptors is poorly understood. Our goal was to determine the antinociceptive potency of the endogenous cannabinoid 2-arachidonoyl-glycerol (2-AG), and its interaction with endomorphin-1 (EM1) at joint level in the rat inflammation model.

2. Mechanical hypersensitivity was produced by injection of carrageenan (300 μg/30 μL) into the tibiotarsal joint of the right hind leg. The mechanical threshold was assessed by von Frey filaments. 2-AG (3–200 μg), EM1 (100–300 μg) and their combinations in a fixed-dose ratio (1 : 10) were given into the inflamed joint, and the threshold was determined repeatedly for 105 min after the drug administrations.

3. Both ligands produced dose-dependent anti-hyperalgesia, and the highest doses caused prolonged effects, but they did not influence the degree of oedema and the withdrawal threshold at the non-inflamed side. EM1 had lower potency compared to 2-AG (ED_{25}: 233 (CI: 198–268) μg and 126 (CI: 88–162) μg, respectively; $P < 0.05$). The effects of EM1 and 2-AG were prevented by μ-opioid and cannabinoid 1 receptor antagonists, respectively. The ED_{25} value for the combination (98 (CI: 80–112) μg) did not differ significantly from the value of 2-AG; however, the largest dose combination produced a significantly higher effect than the ligands by themselves.

4. Our data showed that 2-AG was an effective antinociceptive ligand at joint level, and its combination with EM1 produced long-lasting, effective antinociception.

Key words: antinociceptive activity, arthritis, cannabinoid, inflammation, interaction, opioid, pain, peripheral sensory neurons.

INTRODUCTION

Chronic pain represents an emerging public health issue of massive proportions, particularly in view of aging populations. Selective influence of peripheral receptors has the important advantage of providing effective analgesia without side effects typically associated with centrally acting drugs. However, in clinical practice, most pain treatment strategies are based on systemic administration of conventional centrally penetrating substances. It is well established that stimulation of central opioid receptors results in analgesia by modulating nociceptive information, but both opioid receptors and endogenous opioids can be widely detected throughout the peripheral nervous system and/or in peripheral tissues.[1–3] Nowadays, the spectacularly developing field of pain research relates to the roles of endogenous ligands. With the discovery of the two endogenous tetrapeptides, endomorphin-1 (EM1) and endomorphin-2 (EM2), highly specific μ-opioid receptor agonists have been identified.[4] A huge amount of data proved the antinociceptive potential of these tetrapeptides at both spinal and supraspinal levels.[5] Considering their peripheral potency, little data is available in this respect.[6–8] In our experiments we used EM1, because some data suggest that EM1 is more efficient than EM2.[9,10]

Cannabinoids (CB) acting on G-protein coupled CB1 and CB2 receptors can also suppress behavioural responses to acute and persistent noxious stimulation.[11] CB1 receptors are expressed mainly by neurons at high levels in different brain areas, in the spinal cord, in neurons of the dorsal root ganglia and in peripheral tissues.[12,13] CB2 receptors occur predominantly peripherally in immune cells, but they have also been found recently in the brain, spinal cord and in the dorsal root ganglia, mainly on glial cells.[11,14–17] Therefore, several data suggest that not only central but also peripheral administration of CB agonists produce antinociception.[11,14] A major limitation to the potential use of CB agonists as therapeutic agents is the profile of side effects, which include dysphoria, effects on motor coordination, memory, and abuse potential. An alternative approach, which might avoid such side effects, is to influence the endogenous cannabinoid system. The family of endocannabinoids contains several polyunsaturated fatty acid derivates, such as N-arachidonoyl ethanolamide (anandamide) and 2-arachidonoyl-glycerol (2-AG).[18,19] A few studies have investigated the antinociceptive potency of 2-AG at the peripheral level. These reports have shown that 2-AG administered intraplantarly inhibits both neuropathic

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Received 8 July 2009; revision 26 November 2009; accepted 30 November 2009.

allodynia and formalin-induced pain behaviour effectively by the activation of CB2 and/or CB1 receptors.[20,21] However, there is no data about its antinociceptive potency after its intraarticular administration. Therefore, the first goal of our study was to determine the antinociceptive potency of 2-AG at joint level in an inflammatory pain model.

A good possibility for overcoming the problems of endogenous ligands (lower affinity and plateau effect) is to use their combinations.[22,23] Many previous studies indicate that both centrally and peripherally administered cannabinoids can enhance the antinociceptive properties of opioids.[24–27] The interaction of endogenous ligands at the peripheral level is still largely unknown. Therefore, the second goal of our study was to examine the interaction of EM1 with 2-AG at the peripheral level.

METHODS

Animals

After the institutional ethical approval had been obtained (Institutional Animal Care Committee of the Faculty of Medicine at the University of Szeged), male Wistar rats (Charles River strain, Bioplan, Budapest, Hungary; 244 ± 2.0 g; n = 8–10/group) were housed in groups of 5–6 per cage, with free access to food and water, and with a natural light : dark cycle.

Drugs

The following drugs were administered: λ-carrageenan, EM1 (Tocris Bioscience, Bristol, UK) and 2-AG (Sigma-Aldrich, Budapest, Hungary), naltrexone (NTX, μ-opioid receptor antagonist; Sigma-Aldrich) and AM 251 (CB1 receptor antagonist; Tocris Bioscience). Carrageenan, NTX and EM1 were dissolved in physiological saline, whereas 2-AG was purchased as a solution diluted in acetonitrile. AM 251 was dissolved in dimethylsulfoxide (DMSO; Sigma–Aldrich) and ethanol as a vehicle, and then it was further diluted with distilled water. The concentration of DMSO and ethanol was 15% and 9%, respectively. Control animals received vehicles or saline. Neither the local nor the systemically administered vehicle-treated groups differed from the saline-treated animals (data are not shown or see Fig. 1c). NTX was administered subcutaneously, while AM 251 was administered intraperitoneally.

Carrageenan-induced inflammation

Inflammation was produced by injecting carrageenan (300 μg/30 μL) into the tibiotarsal joint of the right hind leg. All treatments were given to gently restrained conscious animals using a 27-gauge needle without anaesthesia so as to exclude any drug interaction. These injections did not elicit signs of major distress.

To determine the change of the size of the inflamed joint, we measured the anteroposterior and mediolateral diameter of the paw at the level of the ankle joint with a digital calliper. The cross-section area was calculated by using the formula $a \times b \times \pi$, where a and b represent the radius in the two aspects.

Behavioural nociceptive testing

The threshold for withdrawal from mechanical stimulation to the plantar aspect of the hind paws was determined with logarithmic series of calibrated von Frey monofilaments (SenseLab–Aesthesiometer, Somedic, Sweden), as previously described.[8,28] Prior to baseline testing, each rat was habituated to a testing box with a wire-mesh grid floor for at least 20 min. Von Frey filaments (bending force ranging from 0.064–110 g) were applied in ascending order using steady 1–2 s applications repeated 10 times perpendicularly through the grid floor to the plantar surface of both hind paws of each rat. If paw withdrawal occurred, we evaluated it as a positive response. Only robust and immediate withdrawal responses from the stimulus were considered.

Experimental protocol

After the baseline determination of the joint diameter and the mechanical paw withdrawal threshold (pre-carrageenan baseline value at −180 min), carrageenan was injected. These measurements were performed again 3 h after carrageenan injection (post-carrageenan baseline values at 0 min). EM1 (100–300 μg) and 2-AG (3–200 μg; almost the highest dose possible in this volume), and their combinations in a fixed dose-ratio (10:1; EM1 : 2-AG: 100 : 10, 200 : 20 and 300 : 30 μg) were given into the inflamed joint (30 μL), and mechanical sensitivity was defined at 10, 20, 30, 45, 60, 75, 90 and 105 min following the drug administrations. The control group received physiological saline. We selected the ratio of 1 : 10 for 2-AG + EM1 based on the potency of the single drugs, because the concentration of 2-AG was 10 mg/mL in the original tube; therefore, a 1 : 1 ratio would not have applied in 30 μL volume in higher doses.

Another group of animals was pretreated with NTX (a well-known antagonist on μ-opioid receptors; 4 mg/kg subcutaneously) 20 min before 300 μg endomorphin-1 administration to reveal the role of the opioid receptor activation by EM1. Yet another group of animals was pre-treated with AM 251 (antagonist of CB1 receptors, 1 mg/kg intraperitoneally) 20 min before 200 μg 2-AG injection to determine the involvement of CB1 receptors in the effects of 2-AG.

At the end of the experiment, the joint diameters were measured again. We did not examine the motor behaviour systematically, nor did we quantify it, but the animals' behaviours were observed, and there were no signs of altered behaviour (immobility, flaccidity, excitation or motor weakness). Animal suffering and the number of animals per group was kept at a minimum.

Statistical analysis

Data are presented as means ± standard mean error. Paw withdrawal latencies on the inflamed side were transformed to percentage maximum possible effect (%MPE) by using the following formula:

$$\%\text{MPE} = (\text{observed threshold} - \text{post-carrageenan baseline threshold} / \text{pre-carrageenan baseline threshold} - \text{post-carrageenan baseline threshold}) \times 100$$

Therefore, 100% MPE means perfect relief of allodynia (equivalent to pre-carrageenan baseline value); whereas 0%MPE means that the observed threshold is equivalent to the post-carrageenan baseline value.

The area under the curve (AUC) values was obtained by calculating the area of the %MPE values between 20–90 min to overcome the difficulties of serial measurements.[29] AUC 7500 value would mean the complete relief of hyperalgesia (100% MPE) during the whole period. We observed almost no effects as regards the AUC values after saline treatment (approximatley 29 ± 17.2 in the AUC). The mean AUC values were also used for linear regression analysis (least square method) to investigate the dose-effect curves. The 50% effective dose (ED_{50}) would mean the dose that yielded 50% MPE for the whole period (3500 AUC). However, the AUC values were much lower for these ligands alone; therefore, we determined the 25% of ED (1750; which means approximately a 60-fold increase in the withdrawal threshold compared to the control group). AUC values were examined by one-way analysis of variance (ANOVA). The significance of differences between the various groups was calculated using the Tukey–Kramer test for post-hoc comparison (P-value < 0.05 was considered significant). Statistics were performed by STATISTICA (Statistica, Tulsa, OK, USA), GraphPad Prism (GraphPad software Inc., La Jolla, California, USA) and SPSS (SPSS, Chicago, IL, USA) software.

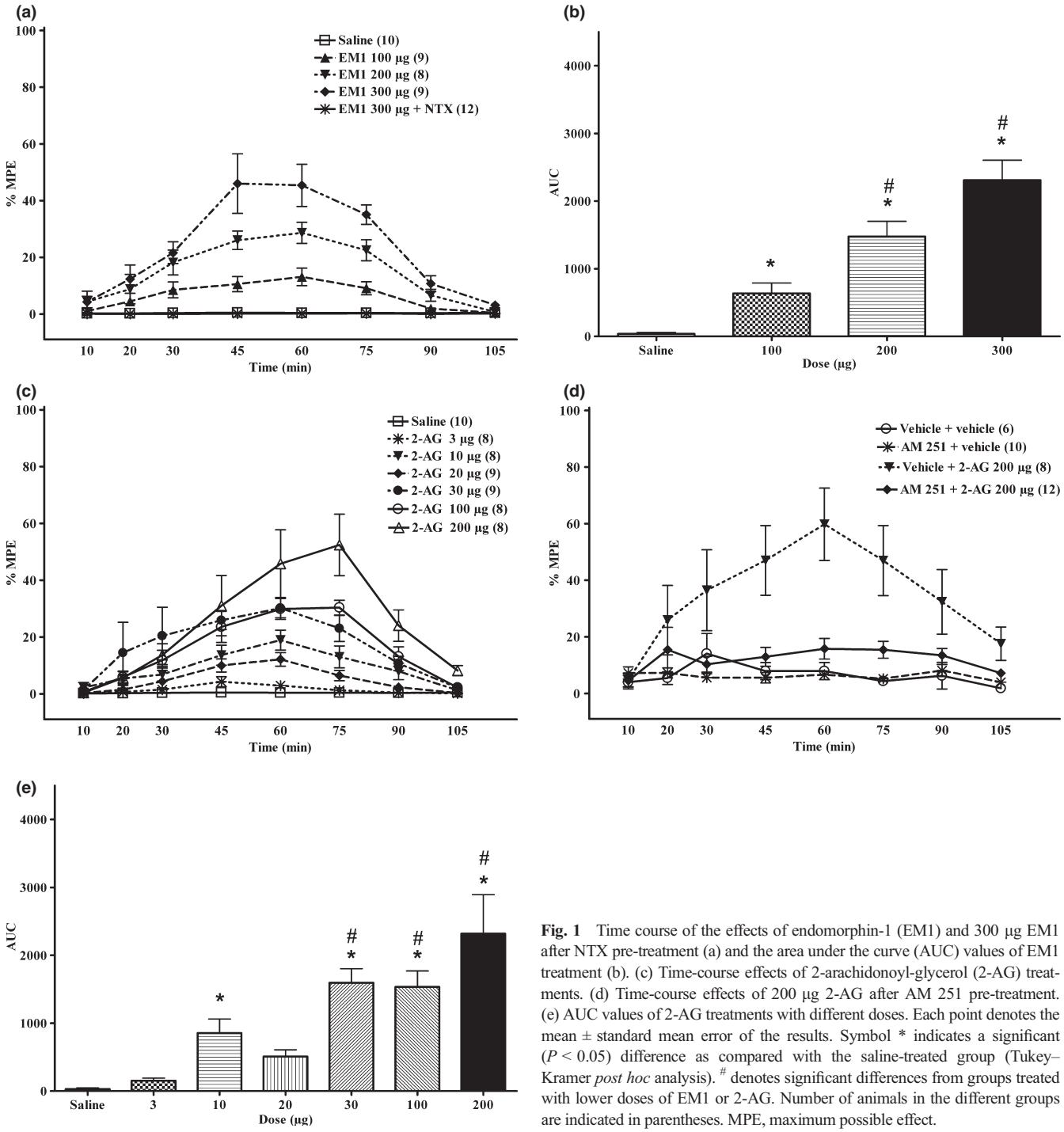

Fig. 1 Time course of the effects of endomorphin-1 (EM1) and 300 µg EM1 after NTX pre-treatment (a) and the area under the curve (AUC) values of EM1 treatment (b). (c) Time-course effects of 2-arachidonoyl-glycerol (2-AG) treatments. (d) Time-course effects of 200 µg 2-AG after AM 251 pre-treatment. (e) AUC values of 2-AG treatments with different doses. Each point denotes the mean ± standard mean error of the results. Symbol * indicates a significant ($P < 0.05$) difference as compared with the saline-treated group (Tukey–Kramer *post hoc* analysis). # denotes significant differences from groups treated with lower doses of EM1 or 2-AG. Number of animals in the different groups are indicated in parentheses. MPE, maximum possible effect.

RESULTS

Joint oedema

There was a significant ($P < 0.01$) increase in joint cross-section area compared with pre-injection control levels 3 h following carrageenan injection into the right ankle, (from 46 ± 0.3 to 75 ± 1.0 mm^2). This conspicuous increase in joint size was a result of oedema formation, confirming the presupposition that carrageenan treatment resulted in an inflammatory reaction. None of the treatments influenced the degree of oedema; the cross-section of the ankle was 76 ± 0.8 mm^2 at the end of the experiments, which did not differ from the post-carrageenan baseline value (to 75 ± 1.0 mm^2).

Mechanosensitivity

Basal mechanical withdrawal threshold was 109 ± 0.5 g (i.e. 96% of the animals did not withdraw their paws at the cut-off value). Carrageenan caused a significant decrease in paw withdrawal threshold on the inflamed side (0.29 ± 0.033 g), but it did not have a significant influence on the non-inflamed side. None of the treatments changed

the mechanosensitivity on the normal side; therefore, results were analyzed only on the inflamed paws.

EM1 produced a dose-dependent antinociceptive effect, which developed gradually, and it reached its maximum between 45 and 60 min (Fig. 1a,b). Therefore, 100 μg EM caused approximately 10 %MPE, whereas 300 μg caused a prolonged effect, which was approximately 50%MPE at the 45th and 60th minute. ANOVA of AUC values showed significant differences between the groups, revealing the dose-dependent effect of EM1 ($F_{3,56} = 41.86$, $P < 0.0001$; Fig. 1b). The ED_{25} AUC value was 233 (CI: 198–268) μg. NTX pretreatment alone did not influence the pain threshold (data are not shown), but prevented the anti-allodynic effect of EM1 (300 μg) (Fig. 1a).

2-AG alone caused a dose-dependent antiallodynic effect, which also developed slowly (Fig. 1c,e). The highest dose produced prolonged antinociception, and it achieved 55%MPE at the 75th min. (Fig. 1c). ANOVA proved that the effects of the treatment were significant ($F_{6,98} = 19.22$, $P < 0.0001$; Fig. 1e). Its potency was higher compared with EM ($P < 0.05$) (i.e. the ED_{25} AUC value was 126 (CI: 88–161) μg). AM 251 pretreatment alone did not influence the pain threshold, but it prevented the anti-allodynic effect of 2-AG (200 μg) (Fig. 1d).

Regarding the interaction of these ligands, co-administration of 10 μg 2-AG with 100 μg, EM1 did not show significant differences compared to the single treatments (Fig. 2a,d). ANOVA revealed significant effects of treatment regarding the 20–200 μg 2-AG–EM1 combination ($F_{3,56} = 48.94$, $P < 0.0001$). Post-hoc comparison showed that this combination produced an increased antinociception compared to vehicle, 2-AG and EM1 (Fig. 2b,e). Similarly, 30 μg 2-AG with 300 μg EM1 also produced long-lasting and more effective antinociception compared to the single treatments ($F_{3,56} = 64.42$, $P < 0.0001$; Fig. 2c,f).

As the ratio of the ED_{25} values of 2-AG/EM was 0.54, the doses of the combinations were calculated in this proportion.[30] The dose–response curve of the cocktail became steeper compared to the EM and 2-AG lines (Fig. 3). The ED_{25} value was 98 (CI: 80–112) μg, which did not differ significantly from the ED_{25} value for 2-AG, suggesting an additive interaction. Because the curve for the combination was steeper compared to the single treatments (using covariant analysis for difference in slope; $P < 0.0001$), the largest dose combination produced significantly higher effect than the ligands alone. No treatment produced motor impairments or other visible adverse effects.

DISCUSSION

Previous studies indicate that exogenous cannabinoids and opioids produce an additive or synergistic interaction at peripheral and central levels.[27,31,32] These studies have made us raise the question of whether a similar interaction might exist between EM-1 and 2-AG at the peripheral level. Our study showed that the intraarticularly administered EM1 and 2-AG dose-dependently decreased mechanical allodynia without effect on the oedema. Mechanical threshold did not change on the non-injected side, suggesting that the intraarticularly injected endogenous ligands did not produce systemic effects in these doses. The antinociceptive effect of EM1 or 2-AG was prevented by systemic NTX or AM 251 pretreatment. The co-administration of EM-1 and 2-AG produced an additive interaction; however, the dose-response curve of the combination was steeper compared to the single treatments, suggesting an even more beneficial effect at higher dose-ranges. We could not exclude completely that the injection of carrageenan into awake animals influenced our results, at least partially. Carrageenan was applied 3 h earlier than the performance of the experiments; therefore, we did not presume that the injection procedure had significant effects on our results. Furthermore, the introduction of anaesthesia might produce stress responses that could also modify the results.

The use of cannabinoids for the management of a wide range of painful disorders has been well documented at spinal, supraspinal and peripheral levels.[11,14,33] Peripheral nerve fibres express CB1 and CB2 receptors and their activation can inhibit pain sensation, and the peripheral immune cell CB2 receptor stimulation may downregulate inflammation by suppressing the release of inflammatory mediators.[11,34] Therefore, topically applied cannabinoids have provided effective analgesia in different pain models, and this effect is mediated by CB1 and CB2 activation.[12,35] The antinociceptive properties of endogenous cannabinoids have been established in a number of experiments, but only a few studies suggest that these ligands can also be involved in the peripheral pain mechanisms.[35–38] Therefore, intraplantar administration of anandamide has reduced hyperalgesia induced by carrageenan or pain behaviour induced by formalin injection by activation of CB1 and CB2 receptors.[36,37] The cannabinoid receptor system presents in the synovium; hence, it might be an important therapeutic target for the treatment of pain and inflammation associated with osteoarthritis and rheumatoid arthritis, but only a few studies have investigated their effects at joint level.[39] Thus, a selective CB1 receptor agonist, arachidonyl-2-chloroethylamide, has been able to reduce the mechanosensitivity of afferent nerve fibres in control and osteoarthritic rat knee joints.[40] A close intra-arterial injection of anandamide to the medial articular nerve has significantly increased the discharge of C-fibres by activation of transient receptor potential vanilloid-1 receptors (TRPV1), both in normal and arthritic rats.[41] Another study has shown that anandamide produced dose-dependent increases in the blood flow of the rat knee joint, when being applied on the surface of the joint.[42] Anandamide can activate the cannabinoid and the TRPV1 receptors, and both of these effects can influence the pain threshold.[43]

The second endocannabinoid identified was 2-AG. This 2-acylglycerol ester is the most abundant endogenous cannabinoid, and its concentration in the brain is 50–500-fold higher than that of anandamide, and it has also been identified peripherally.[12,44] 2-AG is rapidly formed from arachidonic acid-containing phospholipids through increased phospholipid metabolism, such as enhanced inositol phospholipid turnover, in various tissues and cells upon stimulation. 2-AG is being inactivated mainly by a monoacylglycerol lipase, but fatty acid amide hydrolase can also inactivate it.[45] 2-AG is a full agonist for CB1 and CB2 receptors with no direct binding to the TRPV1 receptor.[19,46] It is also a substrate for cyclooxygenase-2 (COX-2), and 2-AG is capable of suppressing elevation of COX-2 expression by activating the CB1 receptors.[47,48] Few studies have proved the antinociceptive potency of 2-AG. 2-AG ($ED_{50} = 12.5$ mg/kg) has caused antinociception in acute pain tests when systemically administered to mice, as well as immobility, reduction of spontaneous activity and lowering of rectal temperature.[19,49] Intraplantarly injected 2-AG (0.01–100 μg) has decreased pain behaviour in a dose-dependent manner in the formalin test, and the antinociceptive effects of 2-AG have been prevented by AM630, a selective CB2 antagonist, but not by AM 251, a selective CB1

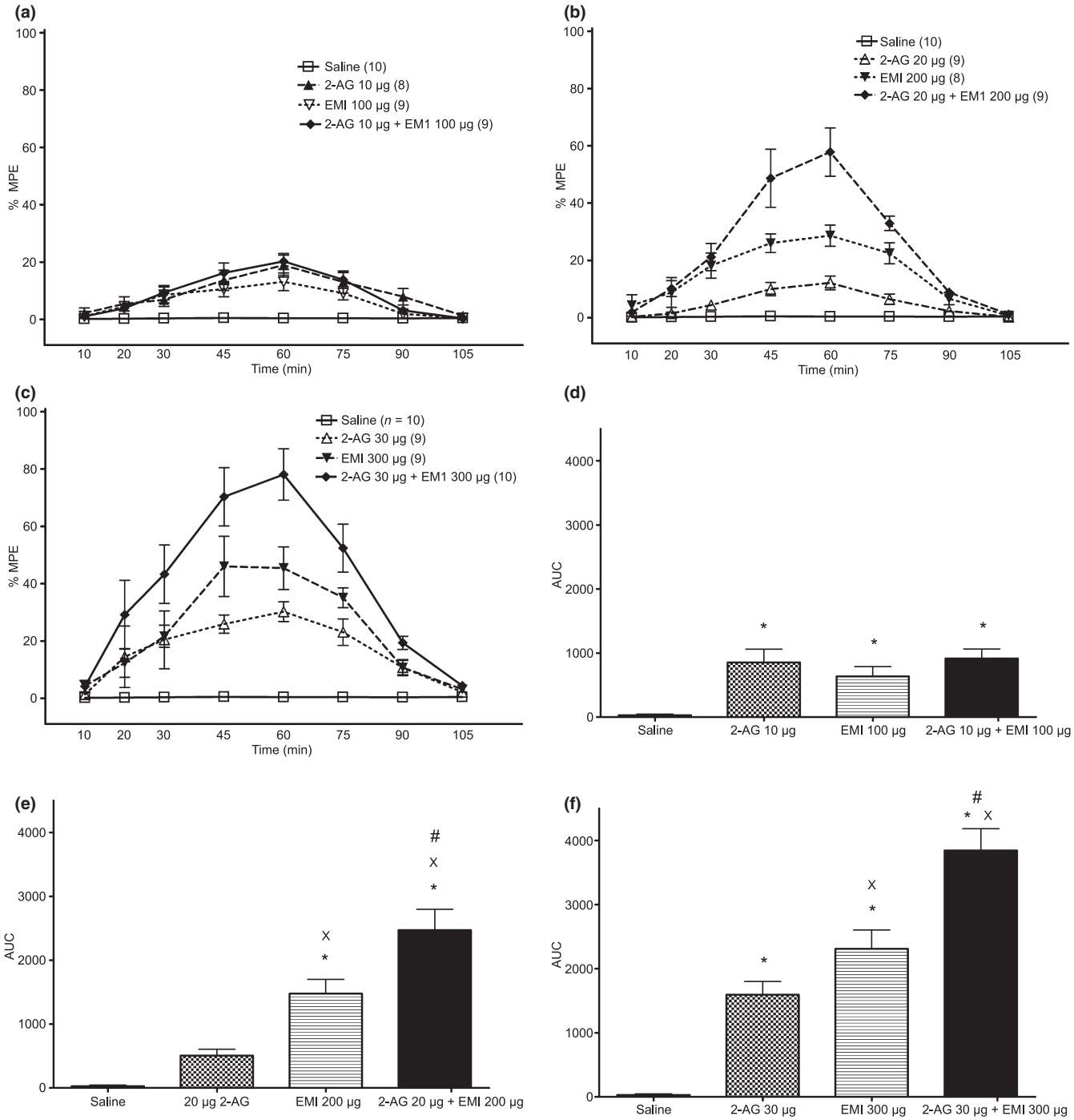

Fig. 2 Time course of the antinociceptive effects and area under the curve (AUC) values of endomorphin-1 (EM1) and 2-arachidonoyl-glycerol (2-AG) in different doses by themselves and in combinations (10 : 1 fixed dose-ratio). Each point denotes the mean ± standard mean error of the results. Symbol * indicates a significant ($P < 0.05$) difference compared to the saline-treated group (Tukey–Kramer post-hoc analysis). # denotes a significant difference from EM1 treated groups. × denotes a significant difference from 2-AG treated groups. Number of animals in the different groups are indicated in parentheses. MPE, maximum possible effect.

receptor antagonist.[21] In contrast, the local administration of 2-AG (10 µg) has significantly decreased mechanical allodynia and thermal hyperalgesia in a neuropathic pain model through the activation of both CB1 and CB2 receptors.[20] Therefore, both of these studies have proved the potency of 2-AG at the peripheral level. In our circumstances, higher doses of 2-AG were effective, which might be due to the differences between the pain models (neuropathy versus carrageenan-induced arthritis). Furthermore, in our model, the CB1 antagonist drug perfectly reversed the effect of the highest dose of 2-AG, suggesting that this antiallodynic effect of 2-AG was mainly due to the activation of CB1 receptors at joint level.

It is well known that locally released opioid peptides at the site of injury can inhibit the inflammatory response, and reduce the pain associated with it.[3] Only a few studies have supported the beneficial

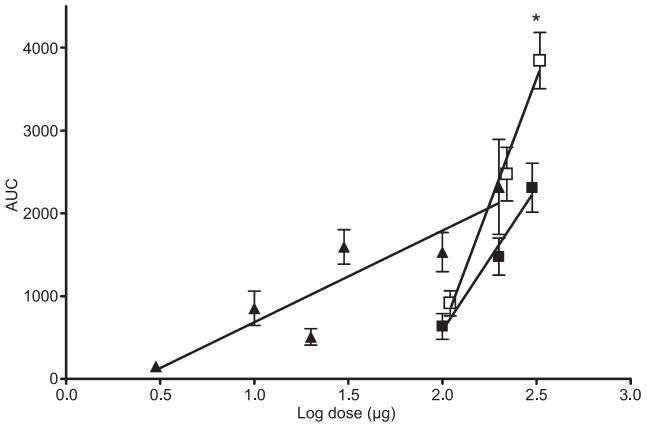

Fig. 3 The magnitude of the dose-dependent effects of endomorphin-1 (EM1) (■), 2-arachidonoyl-glycerol (2-AG) (▲) by themselves and their combinations (□) (area under the curve (AUC) values between 20 and 90 min). Symbol * indicates a significant ($P < 0.05$) difference compared to the group treated with 200 μg 2-arachidonoyl-glycerol (2-AG) and EM1 by themselves.

effects of EM1 at peripheral level. Therefore, intraplantar administration of 33–262 nmol (20–160 μg) EM1 has decreased the mechanical allodynia and the thermal hypersensitivity in a neuropathic pain model dose-dependently.[6] Others have found that the intraplantarly applied EM1 in low doses (0.3–1.25 μg) decreases Freund's adjuvant-induced mechanical allodynia.[7] As regards the role of endomorphins at the joint level, EM1 has been identified in the synovial tissue in humans but not in rats with normal joints; however, articular inflammation has significantly increased its level in patients and animals.[9,50] The intraarticularly administered EM1 reduces the blood flow of the knee joint through its action on unmyelinated primary afferent neurons, and this effect is not sustainable during advanced inflammation.[51] The loss of this response appears to be due to down-regulation of μ-opioid receptors as a consequence of EM1 accumulation within the arthritic joint. EM1 can also decrease the joint inflammation, and this effect might contribute to its antinociceptive potency.[9] EM-1 has reduced normal knee mechanosensitivity to a noxious stimulus after close intraarterial injection to the knee joint, whereas its analgesic effect has been lost during chronic inflammation.[10] Our earlier results showed that intraarticular administration of EM-1 into the tibiotarsal joint significantly decreased the mechanical allodynia in the carrageenan-induced inflammatory pain model.[8] Considering the action mechanism of EM1, it is suggested that the activation of μ-opioid receptors by EM1 (because it was reversible by a μ-opioid antagonist) can inhibit the release of pain producing substances (e.g. substance P) from primary sensory neurons.[5]

Although the beneficial interaction between opioids and cannabinoids are well known after systemic and/or central administrations,[24,52] only a few studies have investigated their interaction at the peripheral level.[53,54] These studies have applied synthetic drugs using a topical immersion method, and the acute heat pain threshold has been determined. It has been found that topically applied cannabinoid potentiates the effect of morphine. We found that the co-administration of endogenous opioid and cannabinoid ligands produced an additive interaction, and they effectively decreased the inflammatory allodynia in our model. Because both μ-opioid and CB receptors can be found in the synovial membrane, and/or on the nociceptive primary sensory neurons, and these receptors are G-protein coupled ones, their co-activations can modify the level of cyclic adenosine monophosphate and phospholipase C, open potassium channels and close calcium channels, and inhibit the release of substance P and other pain-inducing ligands leading to a potentiated inhibition of the propagation of nociceptive stimuli.[3,39,55,56]

In conclusion, these findings are the first to demonstrate that intra-articularly injected 2-AG and its combination with EM1 decreased pain behaviour in a model of arthritic pain. Therefore, interaction of these endogenous substances, acting at different receptor types, might be beneficial in attenuating the inflammatory pain hypersensitivity.

ACKNOWLEDGEMENTS

The professional technical assistance of Agnes Tandari and Zita Petrovszki is gratefully acknowledged. This study was supported by a grant of the National Research and Development Office, Hungary (OMFB-0066/2005/DNT) and the Hungarian Research Grant (OTKA, K60278).

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