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The antinociceptive potency of N-arachidonoyl-dopamine (NADA) and its interaction with endomorphin-1 at the spinal level

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ABSTRACT

The endogenous N-arachidonoyl-dopamine (NADA) activates both transient receptor potential vanilloid1 (TRPV1) and cannabinoid-1 (CB_1) receptors. The goal of this study was to characterize the antinociceptive potential of NADA on inflammatory thermal hyperalgesia in rats at spinal level, and to determine its interaction with endomorphin-1 (EM) at the spinal level.

The effects of NADA and EM on thermal hyperalgesia were evaluated in rats with a unilateral hind paw carrageenan-induced inflammation. Intrathecal injection of either EM (0.03–10 μg) or NADA (1.5–50 μg) caused dose-dependent antihyperalgesia, but NADA was 5.4 times less potent than EM. The antihyperalgesia caused by 15 μg NADA was inhibited by the TRPV1 antagonist AMG9810, but not by CB_1 antagonist/inverse agonist AM 251, whereas the effect of 50 μg NADA was decreased by both drugs. Co-administration of EM with NADA in 1:15 and 1:50 ratios produced a short-lasting potentiation, but isobolographic analysis for the whole investigated period revealed additive interaction between the two endogenous ligands.

The results show that both TRPV1 and CB_1 receptor activation play a substantial role in the antinociceptive effects of NADA at spinal level, while co-administration of NADA with EM did not show potentiation.

1. Introduction

Both natural and synthetic cannabinoids (CB) potently reduce pain-related behavior in different pain models, and CBs are comparable with opiates in both potency and efficacy (Hohmann, 2002; Pertwee, 2001; Walker et al., 2002). A major limitation for the potential use of CB agonists as therapeutic agents is the profile of side effects, which include dysphoria, dizziness, effects on motor coordination, memory and abuse potential (Carlini, 2004; Gardner, 2005). An alternative approach, which may avoid such side effects, is to influence the endogenous CB system. Most of the endocannabinoids are lipid derivatives and their physiological properties have been the target of several studies. Some of these agents (e.g. anandamide) bind not only to the cannabinoid receptors, but also possess agonist properties at the capsaicin- and heat-activated transient receptor potential vanilloid1 (TRPV1) channel (Chu et al., 2003; De Petrocellis et al., 2004; De Petrocellis et al., 2000). N-arachidonoyl-dopamine (NADA) was identified as an endogenous ligand at both TRPV1 and cannabinoid CB_1 receptors (Bisogno et al., 2000; Chu et al., 2003; De Petrocellis et al., 2004; Huang et al., 2002; Toth et al., 2003). This arachidonic acid derivative was found in the highest concentrations in the striatum and hippocampus, and was also detected in the cerebellum, thalamus and dorsal root ganglion (DRG) (Huang et al., 2002). It is the first endogenous compound identified in mammals that is almost equipotent to capsaicin at TRPV1 receptor (Huang et al., 2002). In addition, NADA also behaves as an agonist at CB_1 receptor with an affinity similar to that of anandamide, the first identified endocannabinoid (Devane et al., 1992). Some studies have shown its effects on pain threshold after different routes of administration (Bisogno et al., 2000; Huang et al., 2002; Huang and Walker, 2006; Pitcher et al., 2007; Price et al., 2004). NADA elicited analgesia following systemic administration (Bisogno et al., 2000). It caused nocifensive behavior when applied on the cornea, and it produced hyperalgesia when administered into the plantar skin of the hind paw (Huang et al., 2002; Price et al., 2004). As regards its effects after intrathecal (IT) administration, Pitcher et al.(2007) have found that NADA produced mechanical allodynia in mice. Our earlier data showed that TRPV1 receptor activation plays a substantial role in the antinociceptive effects of anandamide at spinal level (Horvath et al., 2008). Since NADA is more potent at TRPV1 than anandamide (De Petrocellis et al., 2000), it might be a more suitable endogenous ligand for the investigation of the role of TRPV1 receptors in the antinociception. The first goal of this study was to characterize the antinociceptive potential of NADA on inflammatory thermal

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hyperalgesia in rats at spinal level. The second goal was to characterize the role of CB_1 and TRPV1 receptors in the effects of NADA.

There are a number of endogenous ligands that have antinociceptive effects in the central nervous system, thus, it is highly unlikely that they work alone, most likely they rather work in concert in the normal animals. Endomorphin-1 (EM) is a μ-opioid agonist endogenous ligand discovered by Zadina et al.(1997). In contrast to morphine, EM has short-lasting effects and there is also evidence suggesting a plateau effect and acute tolerance (Horvath et al., 1999; Horvath, 2000; Sanchez-Blazquez et al., 1999; Stone et al., 1997). The synergistic antinociceptive interactions between synthetic and plant-originated cannabinoids and opioids have been shown, but only few results are available yet about the interaction of endogenous ligands acting on these receptors (Cichewicz, 2004; Tuboly et al., 2009; Welch and Eads, 1999). Our recent study showed that anandamide potentiated the antinociceptive effects of EM at specific doses following IT administration (Tuboly et al., 2009). Both EM and NADA can influence the spinal dorsal horn neurons since their receptors are available pre- and/or postsynaptically (Coggeshall and Carlton, 1997; Horvath, 2000; Howlett, 2002; Huang et al., 2002). Therefore, the third aim of the present study was to determine the interaction of NADA with EM at spinal level.

2. Materials and methods

2.1. Intrathecal catheterization

The procedures involved in animal surgery and testing were approved by the Institutional Animal Care Committee of the University of Szeged, Faculty of Medicine. Male Wistar rats (239 ± 1.2 g) were anesthetized with a mixture of ketamine hydrochloride and xylazine (72 and 8 mg/kg intraperitoneally, respectively). An IT catheter (PE-10 tubing; Intramedic, Clay Adams; Becton Dickinson; Parsippany, NJ; I.D. 0.28 mm; O.D. 0.61 mm) was inserted via the cisterna magna and passed 8.5 cm caudally into the subarachnoid space (Yaksh and Rudy, 1976), which served to place the catheter tip between Th12 and L2 vertebrae, corresponding to the spinal segments that innervate the hindpaws (Dobos et al., 2003). After surgery, the rats were housed individually and had free access to food and water. Animals exhibiting postoperative neurologic deficits (about 10%), and also those ones that did not show paralysis of one of the hindpaws (about 0.5%) after the administration of 100 μg lidocaine were excluded (Dobos et al., 2003). After the surgery, animals were administered with antibiotic therapy (13 mg/kg gentamicin, subcutaneously) to prevent infection. The rats were allowed to recover for at least four days before testing, and were assigned randomly to the treatment groups (7–12 rats/group). The observer was blinded to the treatment administered.

2.2. Drugs

The following drugs were used: ketamine hydrochloride (Calypsol, Richter Gedeon RT, Budapest, Hungary), xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany), Gentamycin-Chinoin (Sanofi-Aventis, Budapest, Hungary), EM, NADA, AM 251 (CB_1 receptor antagonist/inverse agonist) and AMG 9810 (TRPV1 receptor antagonist) (all were purchased from Sigma-Aldrich; Budapest, Hungary). EM was dissolved in saline, NADA was originally in ethanol solution, and it was diluted with saline, and the final concentration of

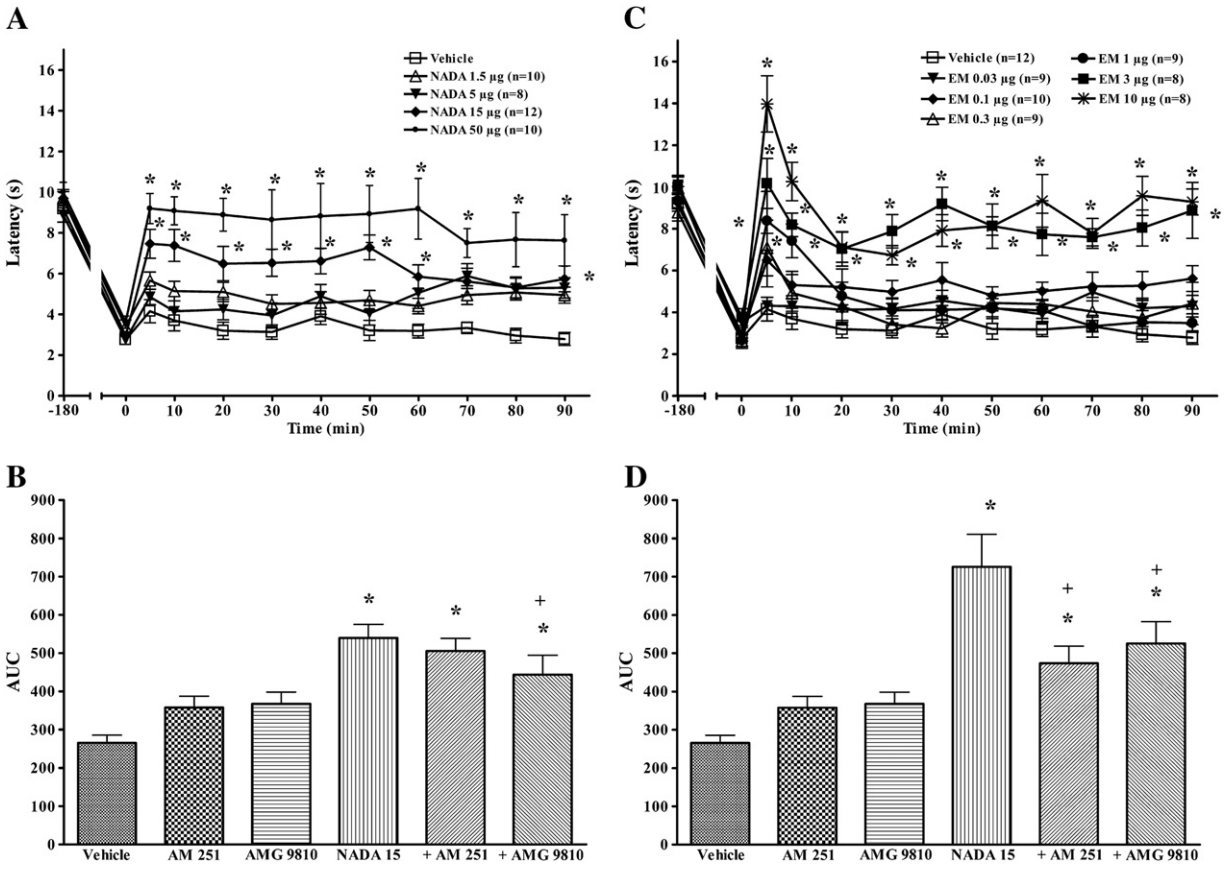

Fig. 1. Time-course effects of NADA (A) and EM (C) and the AUC values the effects of CB_1 (AM 251, 10 μg) and TRPV1 (AMG 9810, 0.3 μg) receptor antagonists on the effects of NADA (15 and 50 μg, B and D, respectively). Each point denotes the mean ± SEM of the results. Symbol * indicates a significant ($p<0.05$) difference as compared with the vehicle-treated group. + denotes a significant difference ($p<0.05$) from NADA treated groups. Number of animals in the different groups is indicated in parentheses.

ethanol was 10%. AM 251 and AMG 9810 were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich, Ltd., Budapest, Hungary) and ethanol and further diluted with distilled water. The final concentration of DMSO and ethanol was 15% and 9% respectively. IT administered drugs were injected over 120 s in a volume of 10 µl, followed by a 10 µl flush of physiological saline. Our preliminary experiments did not show significant differences between the different vehicle-treated rats, therefore, our control group was formed by the 10% ethanol treated animals.

2.3. Nociceptive testing

The hind paw response latency to a noxious heat stimulus was measured to assess the antinociceptive effects of the substances in rats with a hind paw carrageenan-induced inflammation. A detailed description of this model has been published by Hargreaves et al. (Hargreaves et al., 1988). Briefly, rats were placed on a glass surface in a plastic chamber and were allowed to acclimatize to their environment for 15–30 min before testing. A heat stimulus was directed onto the plantar surface of each hindpaw, and the intensity of the thermal stimulus was adjusted to derive an average baseline latency (the time to withdrawal of the hind paw from the heat source, measured in seconds) of approximately 10.0 s. The cut-off time was set at 20 s to avoid tissue damage. The baseline hindpaw withdrawal latencies (PWL; pre-carrageenan baseline values at −180 min) were then obtained. Unilateral inflammation was induced by intraplantar injection of 2 mg carrageenan in 0.1 ml physiological saline into one of the hindpaws (on the paralyzed side during the lidocaine effect) (Dobos et al., 2003). Carrageenan-induced thermal hyperalgesia peaked at 3–4 h after the injection. PWLs were obtained again 3 h after carrageenan injection (post-carrageenan baseline values at 0 min). NADA, AM251, AMG 9810, EM or the combinations were injected after the determination of the post-carrageenan baseline value. PWLs were registered 5 min after the intrathecal injection and then every 10 min until 90 min.

2.4. Experimental paradigm

The first series of experiments was performed to determine the dose response effect and time course for IT administered NADA (1.5, 5, 15 and 50 µg) and EM (0.03, 0.1, 0.3, 1, 3 and 10 µg). In the second series, 15 or 50 µg NADA were co-administered with antagonist, either AM 251 (10 µg) or AMG 9810 (0.3 µg). The dose of antagonists was based on earlier results (Pitcher et al., 2007; Succar et al., 2007; Trang et al., 2006). The third series of experiments was performed with 1:15 and 1:50 ratios of EM and NADA in order to determine their interaction.

2.5. Statistical analysis

Data are presented as means ± SEM. The area under the curve (AUC) values were obtained by calculating the area between 5 and 90 min following IT-injection to construct dose response curves for different doses of NADA alone and/or with EM. AUC 828 (AUC_{max}) value would mean the complete relief of hyperalgesia (PWL: 9.74 s; the mean pre-carrageenan baseline value) during the whole period. We observed almost no effects as regards the AUC values after saline treatment ($AUC_{min} = 281 \pm 21.6$). The mean AUC values were used for linear regression analysis (least square method) to determine the ED_{50} values with 95% confidence intervals (CI). The 50% effective dose (ED_{50}) would mean the dose that yielded 50% increase in the PWD latency for the whole period ($[AUC_{max} + AUC_{min}]/2 = 554$).

The AUC data sets were examined by one-way analysis of variance (ANOVA), and the time–course curves were analyzed by repeated measurement of ANOVA. *Post hoc* comparisons were carried out with the Bonferroni test.

To determine whether the interaction between the agents is additive or synergistic, isobolographic analyses were used according to earlier results (Tallarida et al. 1989; Ossipov et al., 1997). Tallarida et al.(1989) have provided a statistical interpretation of isobolographic data in which the experimental ED_{50} of a mixture could be compared with a theoretic additive ED_{50}. A line of additive interaction was estimated by connecting the ED_{50} for NADA with EM. For the ratios of drugs used, theoretic additive ED_{50} values can be calculated with the confidence intervals. The theoretic additive points were then compared with the experimentally derived ED_{50} for the mixture by

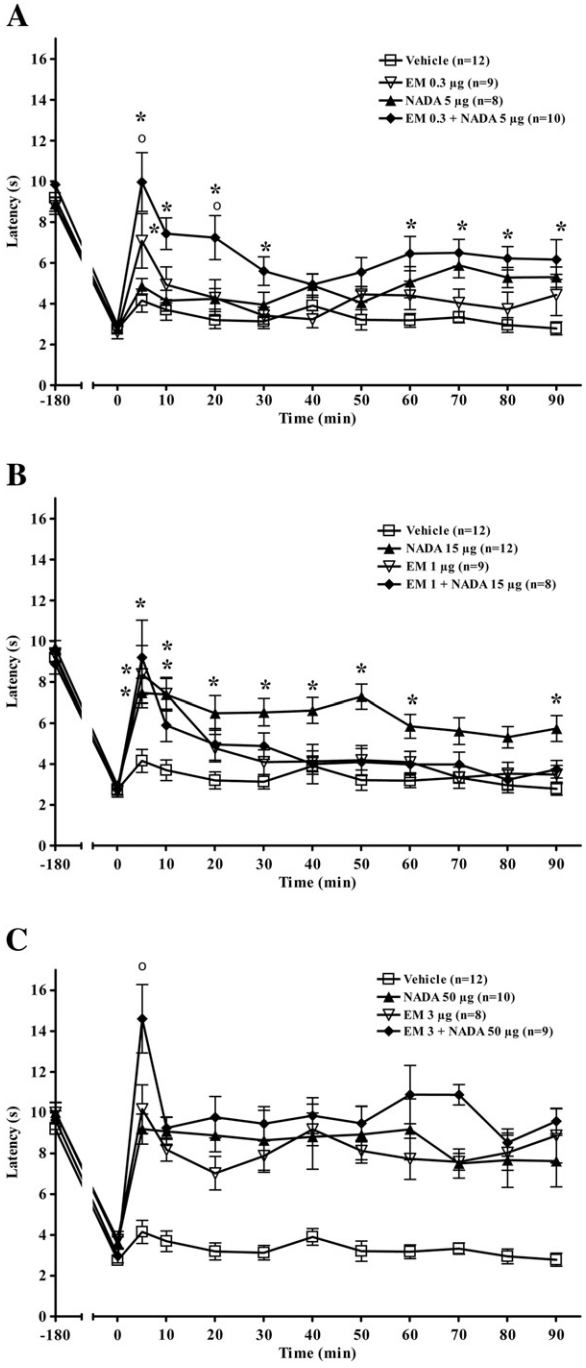

Fig. 2. Time-course effects of different combinations of EM and NADA in 1:15 ratio. Each point denotes the mean ± SEM of the results. Symbol * indicates a significant ($p<0.05$) difference as compared with the vehicle-treated group. ^{O} denotes significant difference from all the other groups. Number of animals in the different groups is indicated in parentheses.

means of a *t* test. A significant potency ratio with the experimental ED_{50} significantly less than the theoretic additive ED_{50} indicates a synergistic interaction.

A *p* value less than 0.05 was considered significant. Statistics were performed by STATISTICA (Statistica Inc., Tulsa, Oklahoma, USA) and GraphPad Prism (GraphPad software Inc. La Jolla, California, USA) software.

3. Results

The basal thermal withdrawal latency was 9.74 ± 0.06 s. Carrageenan caused a significant decrease on PWL at the inflamed hind paw (2.97 ± 0.06 s), whereas the threshold contralaterally did not change significantly (9.67 ± 0.12 s). Higher doses of EM (≥ 1 μg) alone and in combinations produced a short-lasting antinociception (between 5 and 10 min) contralaterally, but no other significant changes occurred (data are not shown), therefore, further analyses were performed ipsilaterally. Neither AM 251 nor AMG 9810 produced any effects compared to the control group (data are not shown).

NADA alone resulted in a dose-dependent effect (Fig. 1A). ANOVA with repeated measurements showed significant effects of treatment ($F_{4,47} = 16.8$, $p<0.001$), time ($F_{11,517} = 34.5$, $p<0.001$), and interaction ($F_{44,517} = 7.2$, $p<0.001$). The highest dose produced a prolonged antihyperalgesic effect—an increase of PWL back to pre-carrageenan baseline. The ED_{50} value was 22.5 (CI: 15.1–30.5) μg for the whole period. However, it should be mentioned that the highest dose (50 μg) also caused temporary vocalization and excitation during injection, suggesting a pain-inducing potential of NADA. With respect to interaction on the CB1 and TRPV1 receptors, AM 251 did not influence the antinociceptive effect of 15 μg NADA, but significantly decreased the effect of 50 μg NADA, while AMG 9810 significantly decreased the effects of both doses of NADA (ANOVA results for AUC with 15 μ NADA: $F_{5,96} = 16.08$, $p<0.0001$; with 50 μg NADA: $F_{5,96} = 17.79$, $p<0.0001$; Fig. 1B and D).

As regards the effect of EM, ANOVA with repeated measurements showed significant effects of treatment ($F_{6,58} = 31.5$, $p<0.001$), time ($F_{11,638} = 164.5$, $p<0.001$), and interaction ($F_{66,517} = 3.3$, $p<0.001$). The lower doses of EM alone caused dose-dependent short-lasting antihyperalgesia at 5th and 10th min, while the higher doses (3 and 10 μg) produced prolonged effects (Fig. 1C). The ED_{50} value was 4.2 (CI: 3.1–5.3) μg for the whole period.

Regarding the interaction of NADA and EM, in ratio 1:15 the lowest dose-combination (EM:NADA = 0.3:5 μg) was effective for almost all the investigated period, while the drugs alone did not produce a pronounced effect (Fig. 2A). Surprisingly, the combination of 1 μg EM with 15 μg NADA produced shortened antinociception compared to NADA treatment (Fig. 2B), while the highest dose-combination was more effective than the ligands alone only in two time points (Fig. 2C). The combinations in 1:50 ratio produced significant antihyperalgesia in dose of 0.1:5 μg (EM:NADA) for 30 min (Fig. 3B) and a higher dose-combination (EM:NADA = 0.3:15 μg) produced a temporary potentiation. In the other two combinations the effects were similar to the NADA treatment alone (Fig. 3A and D).

The dose–response curves and the isobolographic analysis of AUC values of EM-NADA combinations indicated that the experimentally derived ED_{50} values did not differ significantly from the theoretical ED_{50} values in both 1:15 and 1:50 ratios, indicating an additive interaction (Table 1, Figs. 4 and 5).

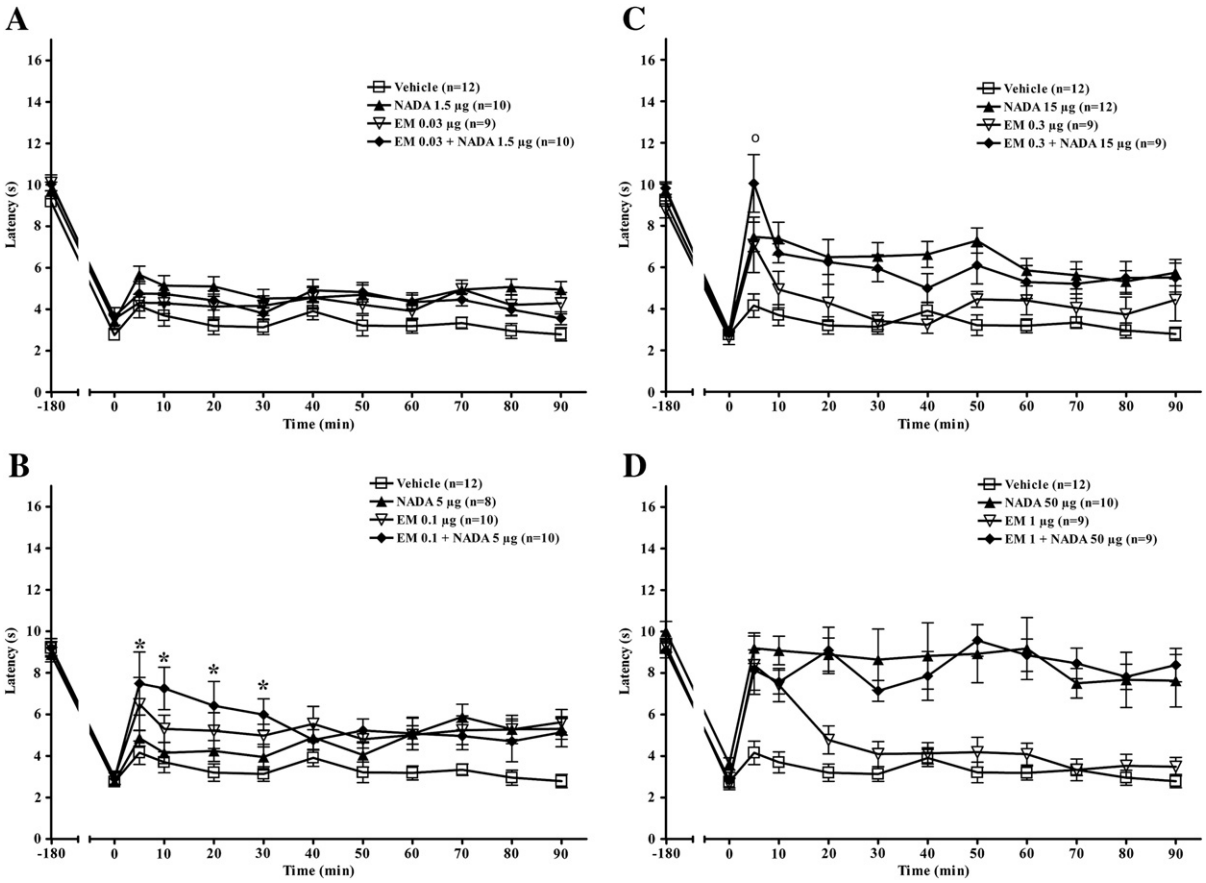

Fig. 3. Time-course effects of different combinations of EM and NADA in 1:50 ratio. Each point denotes the mean ± SEM of the results. Symbol * indicates a significant ($p<0.05$) difference as compared with the vehicle-treated group. ^{O} denotes significant difference from all the other groups. Number of animals in the different groups is indicated in parentheses.

Table 1
Antinociceptive potency of drugs alone and in combinations.

Treatment	Exp. ED_{50} (μg) [CI]	Theor. ED_{50} (μg) [CI]
EM	4.2 [3.1–5.3]	
NADA	22.5 [15.1–30.5]	
EM:NADA = 1:15	19.6 [10.8–28.5]	17.8 [14.3–28.9]
EM:NADA = 1:50	25.2 [18.1–32.5]	20.9 [14.9–30]

Abbreviations: Exp: experimentally derived; CI: confidence interval; Theor: theoretical; EM: endomorphin-1; NADA: N-arachidonoyl-dopamine.

4. Discussion

The results of our study show that IT administration of NADA caused dose-dependent antihyperalgesic effect in the inflammatory pain model. Both CB_1 and TRPV1 receptor antagonists decreased the antihyperalgesic effect of NADA, indicating that these receptors are involved at spinal level. The co-administration of EM produced short-lasting potentiation of NADA antinociception, however, the isobolographic analysis of AUC values for the whole investigated period showed additive interaction.

The dual effects of NADA at TRPV1 and CB_1 receptors have been confirmed in some earlier studies. Thus in vitro application of NADA to DRG neurons and insulinoma ß-cells causes membrane depolarization and a significant increase in intracellular calcium, which are blocked by both TRPV1 and CB_1-receptor antagonists (De Petrocellis et al., 2007; Sagar et al., 2004). NADA leads to the release of substance P and calcitonin gene related peptide (CGRP) from DRG, trigeminal ganglion neurons via activation of TRPV1 receptors (Huang et al., 2002; Price et al., 2004). Furthermore, NADA increases or decreases glutamatergic synaptic transmission to dopaminergic neurons via TRPV1 and CB_1 receptor, respectively (Marinelli et al., 2007). It has also been established that NADA needs to be taken up by cells via the endocannabinoid membrane transporter (EMT) to interact with the TRPV1 receptors. After inhibition of its reuptake, NADA acts as a selective CB_1 agonist suggesting that EMT plays a key role in modulating the stimulation of TRPV1 or CB_1 receptors (Marinelli et al., 2007). It is well known that CB1 receptors are negatively coupled to adenylyl cyclase enzyme through Gi proteins and positively coupled to mitogen-activated protein kinases (Howlett, 2002). The further investigations of these intracellular mechanisms can help in ascertaining the exact role of CB1 receptors in the antinociceptive effects of NADA. NADA also inhibits the activity of fatty acid hydrolase enzyme, and this effect might increase the level of other endogenous cannabinoids (e.g. anandamide) (Bisogno et al., 2000).

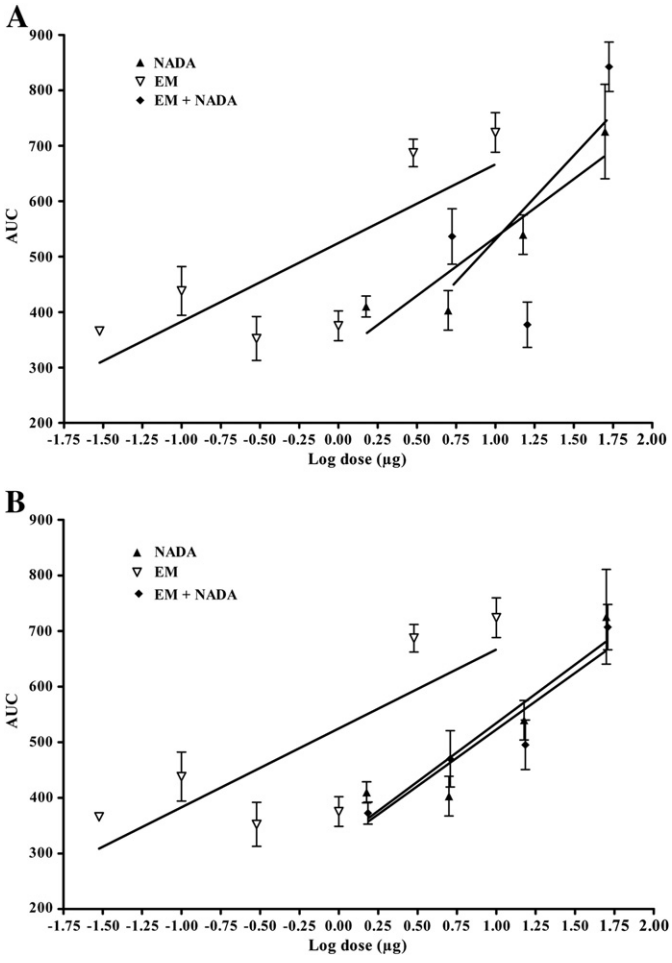

Fig. 4. The magnitude of the dose-dependent effects of EM and NADA by themselves and their combinations (AUC values between 5 and 90 min) in 1:15 (A) and 1:50 (B) ratios.

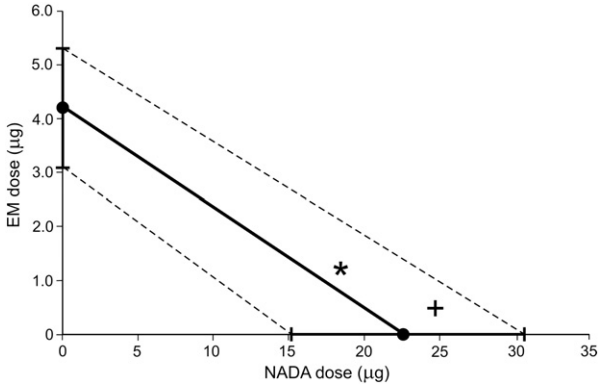

Fig. 5. ED_{50} isobologram (additive line-) with confidence interval (—) for the interaction of EM and NADA. Points shown are the ED_{50} values for EM and NADA alone (•), and the ED_{50} values for combinations in ratios of 1:15 (*) and 1:50 (+).

As regards the in vivo results, intravenous administration of NADA to rats (1-, 4- and 10 mg/kg) induces dose-dependent decreases in mean arterial blood pressure via TRPV1 receptor in rats fed with normal or high sodium diet (Wang and Wang, 2007), while it (10 mg/kg intraperitoneally) causes hypothermia, hypolocomotion and immobility through the activation of CB_1 receptor in mice (Bisogno et al., 2000). Furthermore, its antiemetic effect (1–2 mg/kg intraperitoneally) in ferrets is inhibited by both receptor antagonists (Sharkey et al., 2007). Some data have shown that NADA may play a role in pain modulation, and these effects can also be mediated by CB_1 or/and TRPV1-receptors. Thus, intraplantar administration of NADA (0.1–10 μg) causes thermal hyperalgesia in rats and increases the spontaneous discharge of spinal nociceptive neurons (0.5–5.0 μg) by activation of TRPV1-receptors (Huang et al., 2002; Huang and Walker, 2006). In contrast, Sagar et al. (2004) have investigated the effect of intraplantar NADA on mechanically evoked responses of dorsal horn neurons in anesthetized rats. NADA (5 μg) significantly inhibited the innocuous evoked responses, and this effect was blocked by a CB1 antagonist, while the inhibitory effect of NADA on noxious evoked responses was inhibited by a TRPV1 (but not CB_1) antagonist. In the eye-wipe-assay, topical NADA (0.1%) has resulted in a nocifensive behavior in rats (Price et al., 2004). As regards the results after its systemic administration, NADA (10 mg/kg, intraperitoneally) has induced analgesia in the hot plate test in mice (Bisogno et al., 2000). Spinal administration of NADA (10 nM/4.4 μg) caused mechanical allodynia in mice to hindpaw stimulation with von Frey hairs via TRPV1 receptor (Pitcher et al., 2007), whereas our results showed that NADA decreased the thermal hyperalgesia by the activation of both TRPV1 and CB_1 receptors. It is possible that the differences in this

study are related to differences in the applied models (acute pain vs. carrageenan-induced inflammation) and/or in the stimuli (mechanical vs thermal). Thermal antihyperalgesia caused by 50 μg NADA was blocked by both CB_1 and TRPV1 receptor antagonists, whereas the effect of 15 μg NADA was inhibited only by the application of TRPV1 antagonist. We suppose that in lower doses, NADA activates primarily the TRPV1 receptors, but in higher doses the greater degree of CB_1 receptor activation might also be involved in its antihyperalgesic effect. The role of TRPV1 receptor in different pain syndromes at both peripherally and spinally is well known, however, activation of TRPV1 receptors not only causes nociception and release of proinflammatory neuropeptides (e.g. SP, CGRP) but can also induce the release of endogenous antinociceptive ligands such as beta-endorphin, somatostatin, glycine or GABA leading to analgesic effects (Bach and Yaksh, 1995; Ferrini et al., 2007; Jancso et al., 1985; Jancso and Lawson, 1990; Kanai et al., 2006; Kanai et al., 2007; Pitcher et al., 2007; Szolcsanyi et al., 1998; Yu et al., 2008). The long-lasting effects of NADA suggest that activation of TRPV1 receptors leads to prolonged release of endogenous antinociceptive ligands. However, it cannot be excluded that NADA, similarly to anandamide, can induce acute desensitization of TRPV1 receptors (Lizanecz et al., 2006). Increasing evidence confirms that cannabinoid and vanilloid systems intensely cooperate in different systems, therefore, their interaction should also be considered after NADA administration. At supraspinal level TRPV1 receptor activation has attenuated the anxiolytic effects of phyto-and endocannabinoids by increasing glutamate release in vivo in rats (Campos and Guimarpes, 2009), whereas capsaicin-evoked release of substance P has been increased by CB_1 receptor antagonist in spinal cord slices (Lever and Malcangio, 2002). CB_1 is found primarily on the primary sensory neurons and on both excitatory and inhibitory interneurons in the superficial spinal cord (Ahluwalia et al., 2000; Farquhar-Smith et al., 2000; Hegyi et al., 2009; Pernia-Andrade et al., 2009), and their activation inhibits these neurons and reduces the releases of several transmitters, however, the activation of TRPV1 receptors increases the transmitter releases, as was discussed above (Helyes et al., 2003; Lever and Malcangio, 2002; Richardson et al., 1998). Since NADA represents a “chimeric” ligand acting on both cannabinoid and TRPV1 receptors, and CBs and TRPV1 receptors show coexpression (Binzen et al., 2006), their coactivations can lead to an interaction between them (Mahmud et al., 2009), thus NADA can influence both the antinociceptive and pronociceptive processes. Furthermore, the exogenously administered ligands can also interact with the endogenously released substances during inflammation. Therefore, the complex changes at the level of different inhibitory and excitatory ligands and their effects on several neurons/receptors in the spinal cord might lead to the prolonged antihyperalgesia. Our results showed that in contrast to the well known synergistic antinociceptive interaction of exogenous cannabinoids and opioids, the co-administration of NADA and EM did not produce synergistic interaction in the applied ratios. Further investigations are necessary to reveal the possible causes of this phenomenon, such as in vitro studies regarding intracellular changes, and experiments for the clarification of the pharmacokinetic interaction of these ligands.

In conclusion, the results show that both TRPV1 and CB_1 receptor activation played a substantial role in the antinociceptive effects of NADA at spinal level. This study supports a role for the endogenous cannabinoid, opioid and TRPV1 receptor-related signalling systems in modulating nociceptive transmission in the spinal cord. We found additive interaction between NADA and EM, which in itself does not reveal a functional cross-talk in vivo of these systems within the framework of spinal nociceptive transmission. The complexity of the pro- and antinociceptive mechanisms remains to be cleared but the co-administration of endocannabinoids and endogenous opioids may be beneficial in special dose ranges, however, further experiments with other models are required in human, and with other endogenous ligands in animals.

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