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The antinociceptive interaction of anandamide and adenosine at the spinal level

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ABSTRACT

Both anandamide and adenosine have significant roles in pain mechanisms, but no data are available concerning their interaction at the spinal level. The goal of this study was to determine how adenosine and the adenosine receptor antagonist caffeine affect the antinociceptive effect of anandamide.
The pain sensitivity was assessed by the acute tail-flick test and by paw withdrawal test after carrageenan-induced inflammation. The substances were administered intrathecally to male Wistar rats.
Anandamide alone (1, 30 and 100 μg) dose-dependently decreased the hyperalgesia, however it had low potency in the tail-flick test. Neither adenosine (100 μg) nor caffeine (400 μg) alone changed the pain sensitivity markedly. Their combination caused a short-lasting antihyperalgesia, but it did not influence the tail-flick latency. Both adenosine and caffeine decreased the antihyperalgesic potential of 100 μg anandamide, while adenosine–caffeine pretreatment temporarily enhanced its effect. As regards acute heat pain sensitivity, no combination with anandamide influenced the effect of anandamide.
These findings provide new data concerning the interaction between two endogenous ligands and caffeine. Since these substances may exert effects on several receptors and/or systems, their interaction in vivo must be very complex and the net outcome after their coadministration could not been predicted from the in vitro results.

1. Introduction

Both natural and synthetic cannabinoids (CB) potently reduce pain-related behavior in different pain models (Hohmann, 2002; Pertwee, 2001). Thus, CBs are highly effective against thermal, mechanical and chemical pain and are comparable to opiates in both potency and efficacy (Walker et al., 2002). A major limitation to 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 manipulate the endogenous CB system. Arachidonylethanolamide (anandamide, AEA), an ethanolamine derivative of arachidonic acid, was first isolated from porcine brain and characterized as an endogenous eicosanoid with moderate affinity for the CB1 and CB2 receptors (Devane et al., 1992). Several lines of evidence suggest that AEA also activates other receptors, including the capsaicin-sensitive transient receptor potential vanilloid 1 (TRPV1) (Hajos et al., 2001; Olah et al., 2001; Oz, 2006; Tognetto et al., 2001; van der Stelt et al., 2005; Zygmunt et al., 1999) and some of its effects, including antinociception, may be at least partially due to TRPV1 activation (Di Marzo et al., 2002; van der Stelt and Di Marzo, 2004; Zygmunt et al., 1999).

Adenosine (ADE), originating from adenosine 5-triphosphate, is another modulator of pain signaling. It is well known that the stimulation of its receptors (A_1, A_{2A}, A_{2B} and A_3) modifies pain signaling, and a variety of molecules have been developed to provide analgesia through this non-opioid mechanism (Poon and Sawynok, 1998; Sawynok, 1998). However, the ADE analogs cause a number of side effects and therefore cannot be used for pain therapy, and ADE is only slightly effective in neuropathic and inflammatory pain states, without influencing the normal pain sensitivity (Chiari and Eisenach, 1999; Kekesi et al., 2004a). A recent study revealed that ADE directly inhibits the TRPV1 channel in vitro, which might influence its antinociceptive potential (Puntambekar et al., 2004).

While the antinociceptive interactions of ADE receptor and CB agonists with opioids have been widely investigated (Lavand'homme and Eisenach, 1999; Welch and Eads, 1999), their use in combination with nonopioid drugs is not well established; only a small number of studies have been made of the interactions of ADE or AEA with drugs acting at other receptors or systems (Guindon et al., 2006; Horvath and Kekesi, 2006; Kekesi et al., 2004a,b), and only a few data are available concerning the effects of coactivation of the ADE and CB receptors (Begg et al., 2002; Dar, 2000; Murillo-Rodriguez et al., 2003).

The goal of this study was to determine the interaction of ADE with AEA after intrathecal administration in acute and inflammatory thermal pain models. We also investigated the effects of the ADE receptor antagonist caffeine (CAFF) on the antinociceptive potency of AEA and ADE.

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2. Methods

After institutional approval had been obtained from the Animal Care Committee of the University of Szeged, Faculty of Medicine, male Wistar rats (214±2.2 g) were studied. For spinal drug administration, the rats were surgically prepared under ketamine plus xylazine anesthesia (72 and 8 mg/kg intraperitoneally, respectively). An intrathecal catheter (PE-10 tubing) was inserted through a small opening in the cisterna magna and passed 8.5 cm caudally into the intrathecal space. After surgery, rats were housed individually, and were allowed to recover for at least 4 days before use. Rats exhibiting postoperative neurologic deficits (about 10%), or which did not display paralysis of one of the hindpaws after administration of 100 μg lidocaine via the intrathecal catheter were not used (Dobos et al., 2003). Animals were assigned randomly to the various treatment groups (n=6–15/group) and the observer was blinded to the treatment administered.

The drugs employed were ketamine hydrochloride (Calypsol, Richter Gedeon RT, Budapest, Hungary), and xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany). ADE, CAFF and AEA were purchased from Sigma-Aldrich (Budapest, Hungary). AEA was dissolved in ethanol:Tween=2:1, CAFF in ethanol, and ADE in saline. The stock solutions were diluted with saline to a final concentration of 10% ethanol. The intrathecally administered drugs were injected over 30–60 s in a volume of 10 μl, followed by a 10 μl flush of physiological saline. Vehicle-treated animals (Veh) served as controls. CAFF pretreatment was performed 10 min before ADE pretreatment (as was described by Lee and Yaksh, 1996), while AEA was administered 10 min after ADE injection.

Rats were placed on a glass surface in a plastic chamber and allowed to acclimatize to their environment for 15–30 min before testing, and the baseline hindpaw withdrawal latencies (pre-carrageenan baseline values at −205 min) were obtained. Heat stimulus was directed onto the plantar surface of each hindpaw, and cut-off time was set at 20 s to avoid tissue damage. Unilateral inflammation was induced by intraplantar injection of 1.5 mg carrageenan in 0.1 ml physiological saline into one of the hindpaws (on the paralyzed side during the lidocaine test) (Dobos et al., 2003). Paw withdrawal latencies were obtained again 3 h after carrageenan injection (post-carrageenan baseline values at −25 min). CAFF or Veh was injected after determination of the post-carrageenan baseline value (−20 min), and the second injection (ADE or Veh) was made at 10 min later (−10 min), and the third one (AEA or Veh) at 0 min. The paw withdrawal latencies were registered twice between the injections (at −15 and −5 min), at 5 min after the third injection, and then every 10 min until 70 min.

The acute pain sensitivity was evaluated with tail-flick test. The reaction time was determined by immersing the lower 5 cm portion of the tail in hot water (51.5 °C) until a tail-withdrawal response was observed. The basal latency was 7.2±0.29 s (at −25 min) and the cut-off time was 20 s. CAFF or Veh was injected after determination of the baseline value (−20 min), the second injection (ADE or vehicle) was made at 10 min later (−10 min), and the third one (AEA or vehicle) at 0 min. Tail-flick latencies were recorded at 5, 10, 30 and 60 min after the last injection.

The first series of experiments was performed to determine the dose–response and time course for intrathecally administered AEA (1, 33 and 100 μg) during inflammation. Since our earlier study showed low potency of ADE (Kekesi et al., 2004a,b), we applied pretreatment with a single high dose of ADE (100 μg). The dose of CAFF administered (400 μg) was based on an earlier study (Esser and Sawynok, 2000).

In the second series of experiments ADE and CAFF were coadministered, and ADE and/or CAFF was then applied with different doses of AEA (1, 33 and 100 μg) in order to determine the effects of these drugs on AEA-induced antinociception.

The third series of experiment determined the effects of ADE and/or CAFF on the antinociceptive potential of 100 μg AEA in the tail-flick test.

Data are presented as means±SEM. In the paw withdrawal tests, to reflect the overall changes in pain sensitivity, the area under the curve (AUC) values were obtained (using GraphPad Prism 4.0 program) by calculating the area after the third injection (5–70 min) for different doses of AEA alone, with ADE and/or with CAFF.

The tail-flick latencies were converted to percentage of the maximum possible effect (% MPE) by using the following formula:

$$\%\text{MPE} = ([\text{observed latency} - \textit{baseline latency}]/[\text{cut off time} - \textit{baseline latency}])*100$$

Data sets were examined by repeated measures of ANOVA between −15 and 70 min. Post-hoc comparisons were carried out with the Fisher LSD test. A P value less than 0.05 was considered significant.

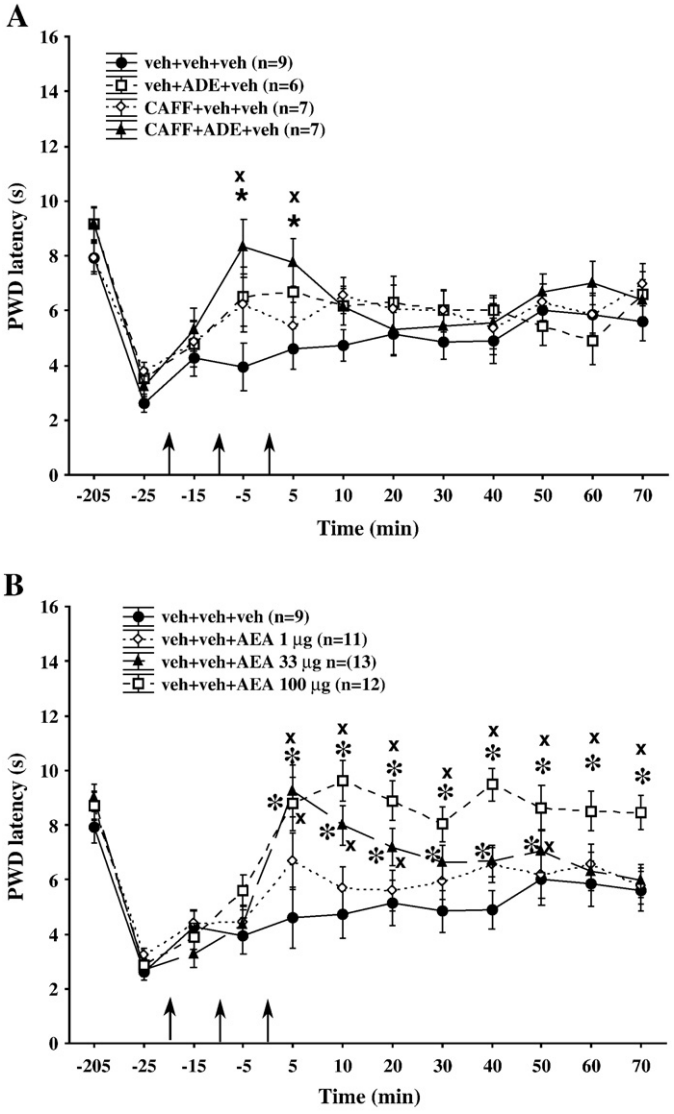

Fig. 1. Time course of the effects of ADE (100 μg), CAFF (400 μg) and their combination on the thermal hyperalgesia (A), and the dose-dependent antihyperalgesic effect of AEA (B). The 1st, 2nd and 3rd arrows show the injection of CAFF/vehicle, ADE/vehicle and AEA/vehicle, respectively. The symbol * denotes a significant (p<0.05) difference as compared with the vehicle-treated group. The symbol x indicates a non-significant difference between the data point and the pre-carrageenan baseline value.

3. Results

3.1. Inflammatory pain sensitivity

Carrageenan drastically decreased the paw withdrawal latency from 8.7±0.15 to 3.3±0.09 s. Because administration of Veh caused a slight decrease in the hyperalgesia, the treated groups were compared to the vehicle-treated one. Since no treatment influenced the normal side significantly (data not shown), we analyzed only the inflamed values.

As regards the effects of ADE (100 μg) and CAFF (400 μg) alone, the comparisons with the control group by two-way ANOVA did not reveal significant differences by treatments (Fig. 1A). ANOVA with repeated measurements revealed a significant effect of time ($F_{9,225}=1.9$, $p<0.05$) and a time×treatment interaction ($F_{27,225}=1.6$, $p<0.05$). The CAFF and ADE cotreatment caused a significant increase in the paw withdrawal latency relative to the control group, and the post-hoc analysis revealed significant differences at −5 and 5 min, suggesting a short-lasting effect of this combination (Fig. 1A), while the AUC analysis did not indicate significant changes (Fig. 2).

AEA caused dose-dependent antihyperalgia (1, 33 and 100 μg), i.e. the lowest dose was ineffective, but the two higher doses increased the paw withdrawal latency significantly, and the highest dose relieved it through the whole of the investigated period (Fig. 1B). ANOVA with repeated measurements revealed significant effects of treatment ($F_{3,41}=7.2$, $p<0.001$), time ($F_{9,369}=16.4$, $p<0.001$), and interaction ($F_{27,369}=2.5$, $p<0.001$). The AUC analysis yielded similar results (Fig. 2A). However, it should be mentioned that the highest dose also caused temporary vocalization and excitation, suggesting a pain-inducing potential of AEA.

Pretreatment with ADE (100 μg) or CAFF (400 μg) did not change the effect of AEA in lower doses (1 and 33 μg) (Fig. 2A). However, the antihyperalgesic potential of 100 μg AEA was decreased both by ADE and by CAFF (Fig. 2A and B). ANOVA with repeated measurements revealed significant effects of time ($F_{9,216}=9.3$, $p<0.001$), and interaction ($F_{18,216}=2.6$, $p<0.001$). The time–response curve demonstrated that the triple combination of AEA 100 μg+ADE+CAFF was more effective than the combination of CAFF+ADE+Veh between 5 and 50 min, but comparison with the AEA-treated group showed a significant difference only 5 min after the last injection (Fig. 2C). ANOVA with repeated measurements showed significant effects of treatment ($F_{2,23}=7.0$, $p<0.005$), time ($F_{9,207}=7.3$, $p<0.001$), and interaction ($F_{18,207}=3.5$, $p<0.001$).

AUC analysis revealed that the results with the triple combination did not differ significantly from those for the AEA-treated animals (Fig. 2A).

3.2. Acute heat pain sensitivity

As regards the acute pain sensitivity, we determined the effects of ADE (100 μg) and CAFF (400 μg) pretreatments on the 100 μg AEA-induced antinociception. In this test neither ADE nor CAFF alone or in combination induced antinociception, only AEA caused a significant, but short-lasting increase in the tail-flick latency, maximally: 46±7.8% MPE at 10 min (Fig. 3A and B). ANOVA did not indicate significant differences between the AEA-containing treatments, suggesting that no combination modified the antinociceptive potential of AEA appreciably (Fig. 3B).

4. Discussion

The results of this study demonstrated that spinal AEA dose-dependently decreased the inflammatory thermal pain sensitivity, and its effects were moderately influenced by ADE, CAFF and their combinations. In contrast, the antinociceptive potency of AEA was not modified by these drugs in the acute thermal pain test.

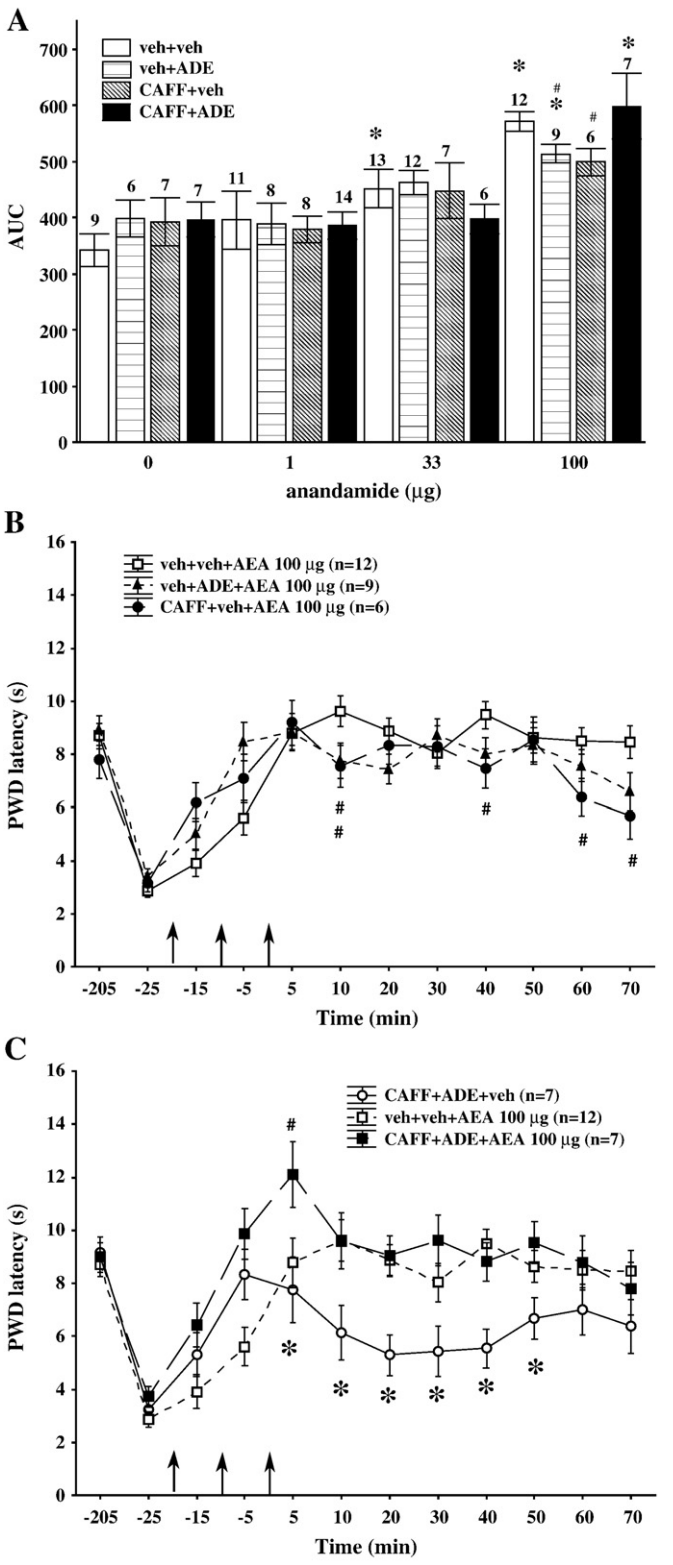

Fig. 2. The magnitude of the effects of different treatments after the drug administrations (AUC values between 5 and 70 min) (A). Time course of the effects of double and triple combinations containing AEA (B, C). The 1st, 2nd and 3rd arrows show the injection of CAFF/vehicle, ADE/vehicle and AEA/vehicle, respectively. The symbol * denotes a significant ($p<0.05$) difference from the correspondence group without AEA. #: significant difference as compared with the AEA treatment group by itself. Numbers above the bars represent *n* values in the groups.

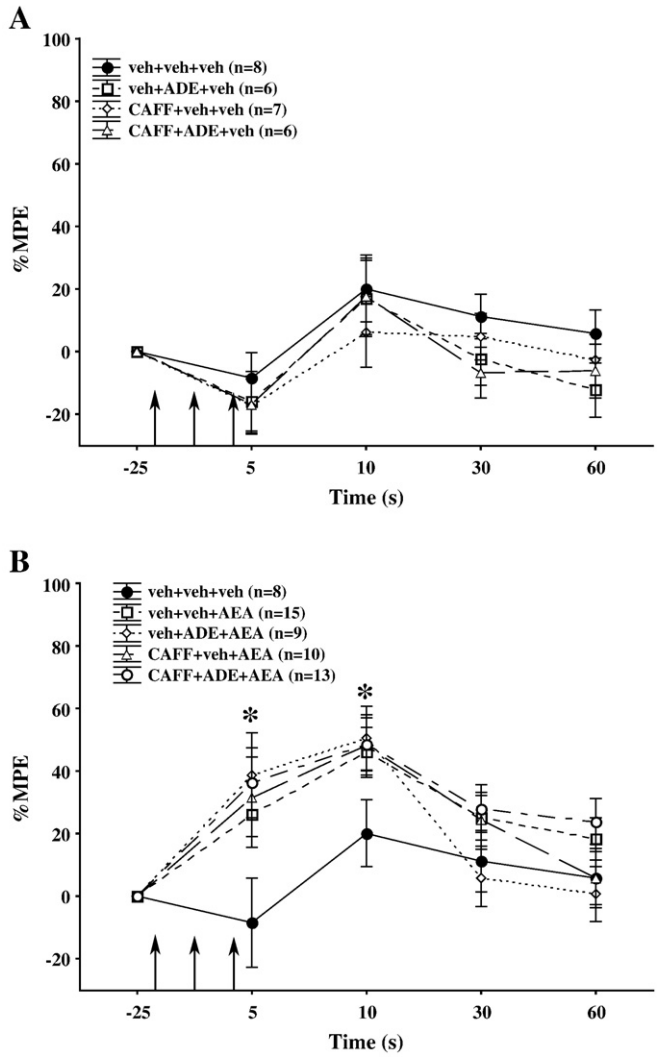

Fig. 3. Time course of the effects of ADE (100 μg), CAFF (400 μg) and their combination (A), and of the double and triple combinations containing AEA (B) on the tail-flick test. The 1st, 2nd and 3rd arrows show the injection of CAFF/vehicle, ADE/vehicle and AEA/vehicle, respectively. The symbol * indicates a significant ($p<0.05$) difference as compared with the vehicle-treated group.

The differences in the results between these two tests might be due to the differences in test circumstances and to the normal vs inflamed situation. It is well-known that the tail-flick but not the paw withdrawal test is performed in a restrained situation which might have influenced the pain sensitivity. Furthermore, the inflammation can induce the release of several endogenous pro- and antinociceptive ligands, which could also have modified the results.

The mechanism of these interactions should be very complex because they may affect multiple receptors pre- and/or postsynaptically (Szallasi et al., 1995; Hohmann et al., 1999; Schulte et al., 2003) (Table 1). First of all, AEA may produce antinociception through the activation of CB1 and CB2 receptors, and CB-receptor antagonists decreased its effects (Ahluwalia et al., 2000; Hohmann et al., 1999; Yaksh et al., 2006). Since microglial activation is also associated with pain and CB2 receptors can depress immune cell activation at the spinal level (Pertwee, 2005; Romero-Sandoval and Eisenach, 2007), it cannot be excluded that the administration of AEA also has antiinflammatory potency, and this may contribute to its antinociceptive effects. As mentioned above, in the case of AEA activation of the TRPV1 receptors must be considered. Both the function and the expression of TRPV1 are enhanced during inflammation (Breese et al., 2005; Kanai et al., 2006; Tohda et al., 2001), and TRPV1 is important for the generation of thermal hyperalgesia however the capsaicin-induced antinociception is also a well-known phenomenon (Dray, 1992; Bolcskei et al., 2005; Caterina et al., 2000). Some data suggest that TRPV1, but not CB1 receptors, are involved in ANA-induced responses in the dorsal root primary neurones in vitro, and it has been suggested that the analgesic properties of AEA are likely to be mediated, at least to some extent, by TRPV1 activation in DRG cells in vivo (Jerman et al., 2002). We observed that that low dose of capsaicin (0.25 μg intrathecally) induced painful behavior during the injection (similar to AEA), but it caused short-lasting analgesia (5–15 min), which was decreased by CAPZ (Horvath et al., 2008). It might be supposed that through the activation of CB1 receptors, AEA at low concentration decreased the transmitter release, while in higher doses it increased the transmitter release via the TRPV1 receptors (Ahluwalia et al., 2003). We presume that the acute activation of primary sensory neurons by high dose of AEA (100 μg) might have caused the short-lasting painful behavior, while the antinociceptive potential of TRPV1 receptor activation might be due to the release of endogenous antinociceptive ligands at spinal level (Bach and Yaksh, 1995; Szolcsányi et al., 1998a,b). Furthermore, TRPV1 receptor activation is also accompanied by activation of the ryanodine receptors causing a further increase in the intracellular Ca^{2+} level (Eun et al., 2001). An additional complication is that AEA acts as a noncompetitive inhibitor of 5HT3 and nicotinic acetylcholine receptors (Oz et al., 2002; Oz, 2006), directly inhibits the voltage-sensitive Na^{+} channels (Kim et al., 2005), and influences the glycine channels (Hejazi et al., 2006; Lozovaya et al., 2005). Moreover, it is likely that other G-protein-related receptors are also involved in some of the actions of AEA observed in CB-receptor knockout mice (Hajos et al., 2001; Oz, 2006). Overall, several systems may be influenced by AEA, and their net effect may be observed under our circumstances.

The low potencies of ADE and CAFF are consistent with earlier results which indicated that ADE was almost ineffective in different pain models (Chiari et al., 1999; Kekesi et al., 2004a,b; Lavand'homme and Eisenach, 1999), while data on the antinociceptive potential of CAFF are controversial and the overall evidence from clinical studies is weak (Camann et al., 1990; Diener et al., 2005). Animal studies have suggested that CAFF induces antinociception, but could inhibit the antinociceptive potential of ADE analogs (Sawynok and Reid, 1996; Sosnowski and Yaksh, 1989). Surprisingly, the coadministration of ADE and CAFF led to a short-lasting antihyperalgesic effect, suggesting some kind of potentiation between them. At first sight this is controversial, however, their interaction might have been complicated

Table 1
Action mechanisms of the ligands

Ligand	G-protein related								Ion-channel				Enzyme
	CB1/CB2	Other	A1	A2a	A2b	A3	Glycine-R	Ryanodine-R	NAch-R	VGNa+	5HT3-R	TRPV1-R	phosphodiesterase
AEA	↑	↑					↓↑		↓	↓	↓	↑	
ADE			↑	↑	↑	↑						↓	
CAFF			↓	↓	↓	↓		↑					↓

↑,↓: activation or inhibition by the ligand, respectively.

by the fact that they influence all types of ADE receptors with different affinities to the receptor subtypes, and studies have demonstrated opposing roles for the receptor subtypes (Patel et al., 2001; Quarta et al., 2004; Ohshita et al., 2007). Furthermore, ADE receptor activation decreases not only the excitatory, but also the inhibitory transmitter release at the spinal level (Yang et al., 2004), which could mask its antinociceptive potential. Stimulation of ADE receptors also inhibits the inflammation (Cronstein, 1994), therefore, this may contribute to its antinociceptive effect. Additionally, the mechanism of action of ADE may be complicated by its interaction with the TRPV1 receptors (Puntambekar et al., 2004). It has been shown that ADE and ADE analogs directly inhibit capsaicin-mediated TRPV1 activation, supporting a role of this nucleoside as an endogenous modulator of TRPV1. In contrast, the activation of TRPV1 in the spinal cord and the periphery promotes the increased release of ADE, possibly through increased intracellular Ca^{2+} entry through the TRPV1 (Sawynok and Liu, 2003; Cahill et al., 1993). CAFF also has several effects on other (nonadenosine receptor-related) systems which might be connected with pain mechanisms (Table 1). Thus, it inhibits phosphodiesterases, leading to elevated levels of cyclic AMP and GMP, and it can also mobilize intracellular Ca^{2+} stores by activation of the ryanodine receptors (Mandel, 2002; Sawynok, 1998). All of these effects could influence the pain sensitivity even in opposite ways (Zupanc et al., 1992; Chung et al., 2003; Galeotti et al., 2005; Yoon et al., 2006). Accordingly, although we expected antagonism between ADE and CAFF, it may be speculated that the potentiation observed might be due to the concurrent influence of the above-mentioned receptors/systems.

As regards their interactions with AEA, we found that both CAFF and ADE decreased the antihyperalgesic effect of AEA, the triple combination caused a temporary potentiation, whereas they did not influence the antinociceptive potential of AEA in the tail-flick test. We suggest that in the case of acute pain sensation, modulation of the above systems does not play a significant role in this respect, or the opposing effects of these systems could counterbalance one another to give a zero net effect. The interactions at the level of the signal transduction pathway on the same synapses or at different synapses are both plausible explanations based on the fact that these receptors are located on several different neuron types in the dorsal horn. The A1 receptor is known to be localized on the same terminals as the CB1 receptors and utilizes the same signal transduction cascade as the CB1 receptors (Ahluwalia et al., 2000; Coggeshall and Carlton, 1997). Since AEA and ADE exert opposite effects on the TRPV1 receptors in vitro, we initially expected that the action of AEA on TRPV1 receptors would be inhibited by ADE. We presumed that after blockade of the ADE receptors (by CAFF), ADE would act mainly as a TRPV1 antagonist, and the triple combination of these drugs would therefore antagonize the effect of AEA on the TRPV1 receptors. Our earlier result that capsazepine decreased the antinociceptive potential of AEA led us to expect a similar results (Horvath et al., 2008). However, the triple combination did not change significantly the effectivity of AEA, which might be due to their multifaceted interactions.

These findings provide new data about the interaction between the endogenous ligands AEA and ADE, and also CAFF. We have found that neither ADE nor CAFF potentiates the antinociceptive effect of AEA at the spinal level in these pain models, and even some kind of antagonism could be found. Further, the coadministration of ADE and CAFF moderately modifies the antinociceptive potential of AEA. We wish to draw the attention to the rapidly evolving recognition that both endogenous and exogenous ligands may exert effects on several receptors and/or systems, therefore we consider that their in vivo interaction must be very complex and the net outcome after their coadministration could not been predicted from the in vitro results. Thus, ADE and AEA cotreatment will presumably not be a beneficial combination for inflammatory pain, but further studies are required in other pain models (e.g. neuropathy) to explore their interactions in pain which is induced by different mechanisms.

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