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Synergistic antinociceptive effects of anandamide, an endocannabinoid, and nonsteroidal anti-inflammatory drugs in peripheral tissue: A role for endogenous fatty-acid ethanolamides?

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit fatty-acid amide hydrolase (FAAH), the enzyme responsible for the metabolism of anandamide, an endocannabinoid. It has been suggested that the mechanisms of action of NSAIDs could be due to inhibition of cyclooxygenase (COX) and also to an increase in endocannabinoid concentrations. In a previous study we have demonstrated that the local analgesic interaction between anandamide and ibuprofen (a non-specific COX inhibitor) was synergistic for the acute and inflammatory phases of the formalin test. To test this hypothesis further, we repeated similar experiments with rofecoxib (a selective COX-2 inhibitor) and also measured the local concentrations of anandamide, and of two fatty-acid amides, oleoylethanolamide and palmitoylethanolamide. We established the ED_{50} for anandamide ($34.52 \text{ pmol} \pm 17.26$) and rofecoxib ($381.72 \text{ pmol} \pm 190.86$) and showed that the analgesic effect of the combination was synergistic. We also found that paw tissue levels of anandamide, oleoylethanolamide and palmitoylethanolamide were significantly higher when anandamide was combined with NSAIDs and that this effect was greater with rofecoxib. In conclusion, local injection of anandamide or rofecoxib was antinociceptive in a test of acute and inflammatory pain and the combination of anandamide with rofecoxib was synergistic. Finally, locally injected anandamide with either NSAID (ibuprofen or rofecoxib) generates higher amount of fatty-acid ethanolamides. The exact comprehension of the mechanisms involved needs further investigation.
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Keywords: Anandamide; Endocannabinoids; Fatty-acid ethanolamides; Oleoylethanolamide; Palmitoylethanolamide; Cannabinoid receptors; Rofecoxib; Ibuprofen; Fatty-acid amide hydrolase; Nonsteroidal anti-inflammatory drugs; Capsazepine; Formalin test; Inflammatory pain

1. Introduction

The principal active ingredient of Cannabis, Δ^9 — tetrahydrocannabinol, produces its effect by binding to G protein-coupled receptors, identified as the cannabinoid CB_1 receptor (Matsuda et al., 1990) localised primarily in the central nervous system (including the spinal cord and dorsal root ganglia) and in the periphery (Rice et al., 2002 for review), and the cannabinoid CB_2 receptor (Munro et al., 1993) mainly expressed in immune tissues (Galiègue et al., 1995). Endogenous cannabinoids (or endocannabinoids) such as anandamide (arachidonylethanolamide), a cannabinoid CB_1 receptor agonist, were also identified (Devane et al., 1992). The therapeutic utility of using compounds that would modulate the endocannabinoid system is beginning to attract more interest (Piomelli et al., 2000). Anandamide can act in the periphery to attenuate pain behaviour. Indeed, when injected into the ipsilateral hind paw of the rat, anandamide reduced hyperalgesia induced by carrageenan (Richardson et al., 1998) or pain induced by formalin injection (Calignano et al., 1998). Those

studies showed that pain relief was produced by the activation of peripheral cannabinoid receptors. Other authors have demonstrated the peripheral mechanisms involved against persistent somatic inflammatory pain (Malan et al., 2001; Nackley et al., 2003).

Anandamide is hydrolysed into arachidonic acid and ethanolamine by a membrane-bound enzyme named fatty-acid amide hydrolase (FAAH) (Cravatt et al., 1996). Anandamide can also be oxygenated by cyclooxygenase-2 (COX-2) to form prostamides, a new class of prostaglandin analogs (Weber et al., 2004; Yang et al., 2005). Other endogenous lipid compounds called fatty-acid ethanolamides also exist such as palmitoylethanolamide and oleoylethanolamide. The former has both anti-inflammatory and antinociceptive properties (Calignano, 1998); it does not bind to cannabinoid receptors (Showalter et al., 1996) although its effects are antagonised by cannabinoid CB_2 antagonists (Calignano et al., 1998). The latter is a naturally occurring lipid that regulates feeding and body weight (Fu et al., 2003; Lo Verme et al., 2005b).

The standard hypothesis for the mechanism of action of nonsteroidal anti-inflammatory drugs (NSAIDs) is by inhibiting COX enzymes responsible for the production of prostaglandins. However, other mechanisms have been proposed, such as interactions with endocannabinoids (Fowler et al., 1997). Indeed, FAAH activity, responsible for the degradation of anandamide, is inhibited by NSAIDs such as ibuprofen, ketorolac and flurbiprofen (Fowler et al., 1997, 1999). However, very little information is available in the literature on the antinociceptive effects of the combination of a cannabinoid with an NSAID. It has been shown that intrathecal indomethacin (Gühring et al., 2002) or flurbiprofen (Ates et al., 2003) is antinociceptive in the formalin test in the rat. This effect was antagonised by a cannabinoid CB_1 antagonist and was absent in cannabinoid CB_1 knockout mice. Furthermore, orally administered palmitoylethanolamide, an endogenous peroxisome proliferator-activated receptor (PPAR)-α agonist with anti-inflammatory properties (Lo Verme et al., 2005a) and indomethacin have demonstrated anti-oedema and anti-inflammatory properties in a rat model of acute inflammation (Conti et al., 2002). Finally in a previous study, we have shown that locally injected anandamide, ibuprofen (a non-specific COX inhibitor) or combination thereof, decreased pain behaviour in the formalin test (Guindon et al., 2006). The combination of anandamide with ibuprofen produced synergistic antinociceptive effects involving both cannabinoid CB_1 and CB_2 receptors. However, the mechanism of this interaction was not explained and the interaction of anandamide with a specific COX-2 inhibitor has not been studied yet. Furthermore, there is evidence of cellular colocalisation of FAAH and transient receptor potential vanilloid 1 (TRPV1) receptors in primary sensory neurons (Price et al., 2005). Therefore, the role of TRPV1 receptors in the context of anandamide/rofecoxib (a selective COX-2 inhibitor) interactions was also evaluated by using a specific antagonist of TRPV1 receptors.

The present study was thus designed to further investigate the antinociceptive interactions between endocannabinoids and NSAIDs. Anandamide and rofecoxib were studied in a test of acute and inflammatory pain. Finally, paw tissue concentrations of fatty-acid ethanolamides were also determined.

2. Methods

2.1. Animals

This research protocol was approved by the Animal Ethics Committee of the Université de Montréal and all procedures conformed to the guidelines of the Canadian Council for Animal Care. Male Wistar rats (Charles River, St-Constant, Québec, Canada), 180–220 g at the time of testing were housed in standard plastic cages with sawdust bedding in a climate-controlled room on a 12-h light/dark cycle. Animals were allowed free access to food pellets and water.

2.2. Drug administration

Anandamide, an endogenous cannabinoid, is a receptor agonist with a four-fold selectivity for the cannabinoid CB_1 receptor (Ki=89 nM) over the cannabinoid CB_2 receptor (Ki=371 nM) and was purchased already in a liquid form in water-dispersible emulsion and further dissolved in 0.9% NaCl (Pertwee, 1999). Capsazepine, a TRPV1 receptor antagonist, was dissolved in 0.9% NaCl solution containing 50% dimethyl sulfoxide (DMSO) (Kwak et al., 1998). Anandamide and capsazepine were purchased from Tocris (Ellisville, MO, USA). Ibuprofen (Sigma, St-Louis, USA), a non-specific COX inhibitor, and rofecoxib, a specific COX-2 inhibitor, were dissolved in 0.9% NaCl solution. For rofecoxib, the compressed tablet from commercial preparations (VIOXX®, 25 mg) was weighed and crushed (using a mortar) into a fine suspension with physiological saline (Francischi et al., 2002). Finally, NS-398 (another specific COX-2 inhibitor) was dissolved in 0.9% NaCl solution containing 4% DMSO and was purchased from Cayman Chemicals (MI, USA).

2.3. Formalin test

The formalin test is a well-established model of inflammatory pain (Tjölsen et al., 1992). Rats were acclimatised to the testing environment (clear Plexiglass box $29 \times 29 \times 25$ cm) during 15 min or until cessation of explorative behaviour. Anandamide (2.88, 8.63, 28.77, 86.31, 288, 2 877 or 14 385 pmol in 50 μl), rofecoxib (31.81, 95.43, 318, 954, 6 362 or 31 810 pmol in 50 μl), NS-398 (159 nmol in 50 μl; Choi et al., 2003), ibuprofen (438 pmol in 50 μl) and capsazepine (2.65 μmol in 50 μl; Kwak et al., 1998) were injected subcutaneously (s.c.) on the dorsal surface of the right hind paw 15 min before the injection (28G needle) of 2.5% formalin (50 μl) next to the previous injection. Following each injection, the rat was immediately put back in the observation chamber. Nociceptive behaviour was observed with the help of a mirror angled at 45° below the observation chamber. Observation of the animal's behaviour was made in consecutive 5-min periods for 60 min following formalin administration. In each 5-min period, the total time the animal spent in three different behavioural categories was recorded: (1) the injected paw has little or no weight placed on it; (2) the injected paw is raised; (3) the injected paw is licked, shaken or bitten. Nociceptive behaviour was quantified using the

composite pain score-weighted scores technique (CPS-WST$_{0,1,2}$ where category 1 score was discarded) calculated for the first (0–15 min) and second (15–50 min) phases of the behavioural response (Watson et al., 1997). The area under the curve (AUC) which corresponds to CPS-WST$_{0,1,2}$ × time (min) was calculated for the acute phase (0–15 min) and the inflammatory phase (15–50 min) using the trapezoidal rule.

2.4. Protocol

The experiments were conducted in a randomized and blinded manner by the same experimenter. In a first study, the dose-response curves for anandamide, rofecoxib and the combination of these two drugs at the fixed 1 to 11 observed ratio given as a total dose and further diluted 10, 30 and 100 times were determined using the data from the inflammatory phase. In a second study, the antinociceptive effects of anandamide were compared under two conditions: absence (0.9% NaCl) and presence of rofecoxib at their ED$_{50}$ doses. In a third experiment, the comparison between two COX-2 inhibitors, NS-398 (159 nmol) and rofecoxib (31.81 nmol) was established. In the fourth experiment, the effects of ibuprofen, anandamide and their combination (at ED$_{50}$ doses taken from our previous study by Guindon et al., 2006) were studied in absence or in presence of capsazepine. In the fifth experiment, the effects of rofecoxib, anandamide and their combination (at ED$_{50}$ doses) were also evaluated in absence or presence of capsazepine. For the first two studies ($n=6$ for each group), in the third study ($n=7$ per group) and in the fourth and fifth studies ($n=4$ per group), the tested drugs were dissolved in the same total volume of 50 µl and administered in the right hind paw. Preliminary experiments in the formalin test ($n=8$ per group) have shown that there was no difference in pain behaviour between 0.9% NaCl and 0.9% NaCl solution with 50% DMSO (data not shown). Therefore, 0.9% NaCl was used as the control solution.

Finally, in order to exclude any possible systemic effect of the drugs, 288 pmol of anandamide, 6.36 nmol of rofecoxib and capsazepine 2.65 µmol (data not shown) were administered s.c. on the dorsal surface of the contralateral (left hind paw) or ipsilateral paws ($n=4$ per group).

At the end of the test (i.e., 60 min after formalin injection), the skin and tissues of the dorsal surface of the right hind paw were removed, flash frozen in liquid nitrogen and stored at −80 °C until used for measurement of fatty-acid ethanolamides as described as followed.

2.5. High performance liquid chromatography /mass spectrometry analysis

2.5.1. Synthesis of [²H₄]-labelled standards

Standard [²H₄]-labelled fatty-acid ethanolamides were synthesised by the reaction of the corresponding fatty acyl chlorides with [²H₄]-labelled ethanolamine. Fatty acyl chlorides (purchased from Nu-Check Prep, Elysian, MN) were dissolved in dichloromethane (10 mg/ml) and allowed to react with 1 equivalent of [²H₄]-labelled ethanolamine (purchased from

Cambridge Isotope Laboratories, Andover, MA) for 15 min at 0–4 °C. The reaction was stopped by adding purified water. After vigorous stirring and phase separation, the upper aqueous phase was discarded and the organic phase was washed twice with water to remove unreacted ethanolamine. The reaction resulted in the quantitative formation of [²H₄]-labelled fatty-acid ethanolamides, which were concentrated to dryness under a stream of N$_2$ and reconstituted in chloroform at a concentration of 20 mM. Fatty-acid ethanolamide solutions were stored at −20 °C until use. Identity and chemical purity (>99.9%) of the synthesised fatty-acid ethanolamides were determined by thin-layer chromatography and high-performance liquid chromatography/mass spectrometry (HPLC/MS).

2.5.2. Tissue preparation

Skin tissue was diced with scissors and incubated in 2 ml of chloroform containing 500 pmol of [²H₄]-labelled-palmitoy-lethanolamide, 500 pmol of [²H₄]-oleoylethanolamide and 25 pmol of [²H₄]-anandamide overnight at +4 °C with shaking. The tissue was then homogenised and lipids were extracted with 2 ml of methanol and 2.25 ml of saturated sodium chloride solution (1 M). The organic layer was removed, evaporated to dryness under N$_2$, reconstituted in a mixture of chloroform/methanol (1:1, 100 µl) and transferred to 2.0 ml screw top vials with 0.1 ml glass inserts to be injected into the HPLC/MS.

2.5.3. HPLC/MS analysis

Fatty-acid ethanolamides were quantified using an isotope dilution HPLC/MS assay in positive ionisation mode (Giuffrida et al., 2000).

2.6. Paw oedema

At the end of the formalin test, paw oedema was measured at the base of the right hind paw using a digital micrometer (Mitutoyo Corporation, USA) with an instrumental error of ±(maximum measuring length/75) µm and a resolution of 0.001 mm (Nackley et al., 2003; Ghilardi et al., 2004).

2.7. Statistical analysis

Pain behaviour for each treatment group was expressed as mean±S.E.M. The dose-response curves for anandamide, rofecoxib and their combination were determined by simultaneous analysis using ALLFIT software (De Léan et al., 1978). Interactions between anandamide and rofecoxib were performed using isobolographic analysis as described by Tallarida et al. (1997) and Grabovsky and Tallarida (2004). The theoretical additive ED$_{50}$ was calculated for the combination of drugs based on the individual ED$_{50}$ and the fixed dose ratio (1:11). For the statistical estimation of the difference between the experimentally derived potency and the theoretical additive counterpart, a t-test was used based on known ED$_{50}$s and standard errors. In the second study, the antinociceptive effects of anandamide in absence or presence of rofecoxib were assessed for significance using a 2 × 2 factorial analysis of variance (ANOVA). The comparison of the two COX-2
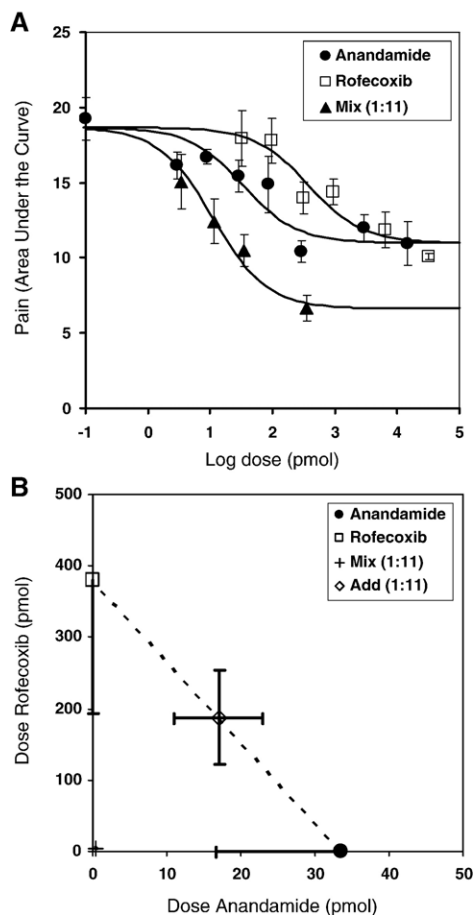
Fig. 1. (A) Dose-response curves for anandamide (full circle), rofecoxib (open squares) and their combination (full triangles) in the inflammatory phase of the formalin test. Data is expressed as mean±S.E.M. (*n*=6). (B) Isobolographic analysis of anandamide/rofecoxib interaction in the inflammatory phase of the formalin test. Drug interactions may be suggested by constructing an isobologram. The ED_{50s} of the two drugs are respectively plotted on the x and y-axes. The straight line connecting these two points is the theoretical additive line. If the experimental derived isobole (a point representing x, y coordinates for ED_{50}) is plotted significantly below the theoretically additive isobole, the interactive effect is identified to be synergistic. Mix 1:11 = equieffective dose of the fixed 1:11 drug mixture; Add 1:11 = expected additive 1:11 combination. The difference between experimental and theoretical points was significant (*P*<0.001) indicating synergistic effect.

inhibitors was assessed for significance using factorial ANOVA. The antinociceptive effects of ibuprofen, anandamide and their combination were assessed for significance in 0.9% NaCl and capsazepine conditions using a factorial ANOVA (Winer, 1971). The same analysis was used for rofecoxib, anandamide and its combination in absence or presence of capsazepine. The differences in the amount of anandamide, oleoylethanolamide and palmitoylethanolamide following the different treatment groups were assessed for significance using factorial ANOVA. To compare ipsi- vs. contralateral paw injections of the drugs, an ANOVA adapted for factorial experimental design was used. The different components of the total variation were settled *a priori* using the multiple regression analysis described by Draper and Smith (1998). The critical level of significance was set at 5% (*P*<0.05).

3. Results

3.1. Synergistic analgesic effects of anandamide and rofecoxib

Anandamide, rofecoxib and their combination decreased pain behaviour in the formalin test with ED_{50} of 34.52 pmol±17.26 (0.012 μg±0.006), 381.72 pmol±190.86 (0.12 μg±0.06) and 11.04 pmol±4.73 (0.0035 μg±0.0015), respectively, and the theoretical additive ED_{50} point was 204.95 pmol±72.52 (0.065 μg±0.023) (Fig. 1). The dose-response curve for anandamide-rofecoxib combination was shifted to the left, away from the anandamide and rofecoxib curves. Isobolographic analysis showed an overall synergistic effect of the combination (*P*<0.001) (Fig. 1A and B).

Pain behaviour following injection of the drugs in the contralateral hind paw was not statistically different when compared with the control group for the acute and inflammatory phases (Fig. 2).

When given locally at ED_{50} doses, anandamide and rofecoxib produced a significant antinociceptive effect both in the acute (*P*<0.001 and *P*<0.025) and the inflammatory phases (*P*<0.001 for both) of the formalin test (Fig. 3).

3.2. Paw oedema

Oedema of the injected paw did not differ significantly among the groups: neither anandamide nor rofecoxib influenced paw oedema (Fig. 3).
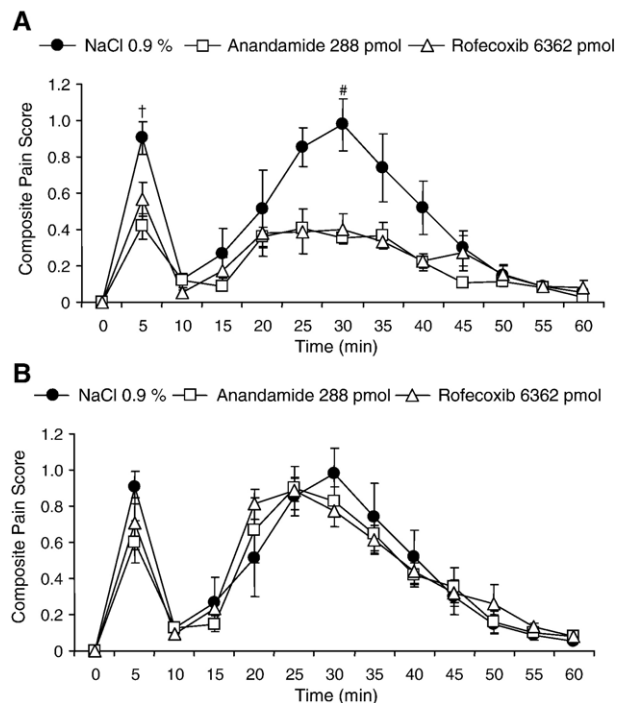

Fig. 2. Effects of anandamide and rofecoxib given on the dorsal surface of the ipsilateral (A) and contralateral (B) hind paws 15 min before 2.5% formalin (50 μl). Data is expressed as mean±S.E.M. (*n*=4). †AUC = Area Under the Curve for the acute phase, *P*<0.01 for analgesics vs. 0.9% NaCl; #AUC for the inflammatory phase, *P*<0.001 for analgesics vs. 0.9% NaCl.
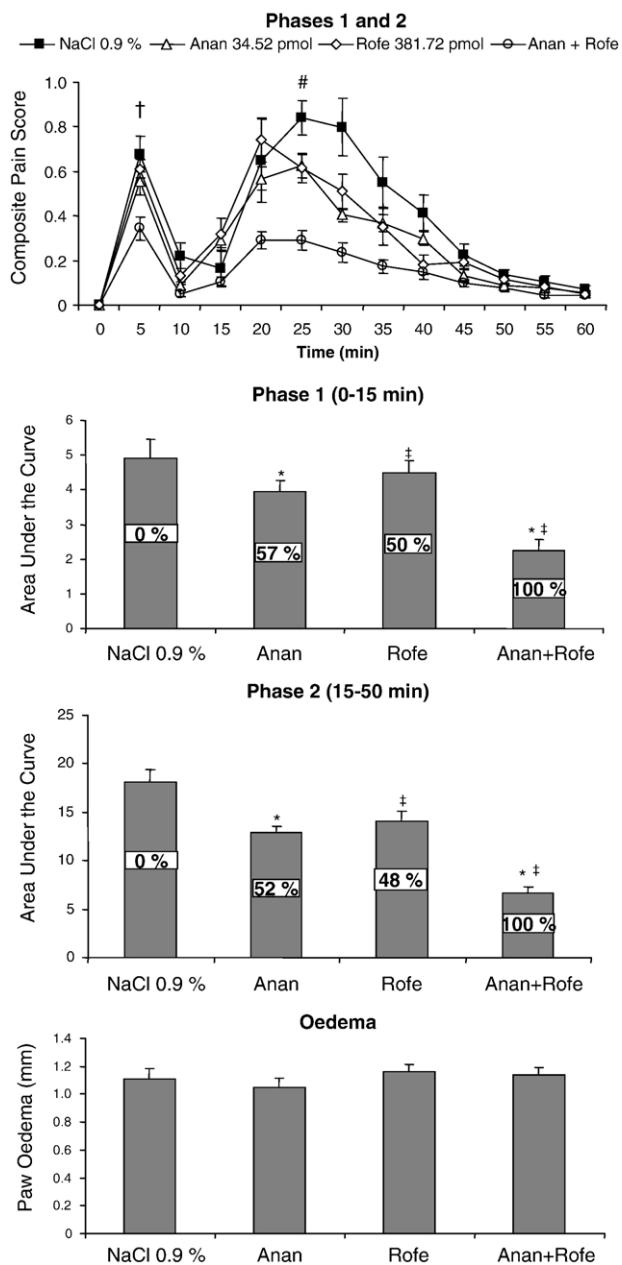
Fig. 3. Effects of anandamide and rofecoxib at ED_{50} doses or their combination given into the hind paws 15 min before 2.5% formalin (50 μl). The upper part of the figure displays the analgesic effects of the drugs compared to control. Phase 1: Additive effect of anandamide combined with rofecoxib for the acute phase of the test (0–15 min). Phase 2: Additive effect of anandamide combined with rofecoxib for the inflammatory phase of the test (15–50 min). Paw oedema. Data is expressed as mean±S.E.M. ($n=6$). Anan = anandamide, Rofe = rofecoxib. † Area Under the Curve (AUC) for the acute phase, $P<0.01$ for analgesics vs. NaCl 0.9%; # AUC for the inflammatory phase, $P<0.001$ for analgesics vs. NaCl 0.9%; * anandamide vs. without anandamide, $P<0.001$; ‡ rofecoxib vs. without rofecoxib, $P<0.025$.

3.3. Comparison of two COX-2 inhibitors: NS-398 and rofecoxib

Local administration of NS-398 was associated with a significant antinociceptive effect. Indeed, when given locally, NS-398 (159 nmol) and rofecoxib (31.81 nmol) produced significant antinociceptive effects compared to the control group in the acute ($P<0.005$) and the inflammatory phases ($P<0.001$) of the test (Fig. 4). However, NS-398 and rofecoxib were not different one from each other for the acute and inflammatory phase, respectively (Fig. 4).

3.4. TRPV1 receptors are not implicated in the antinociceptive effect of the anandamide–ibuprofen combination

Local administration of capsazepine was associated with a significant antinociceptive effect. Furthermore, when given locally at ED_{50} doses, anandamide (51.79 pmol±25.89), ibuprofen (788.44 pmol±394.22), and their combination (25.46 pmol±8.49) (Guindon et al., 2006) alone or in association
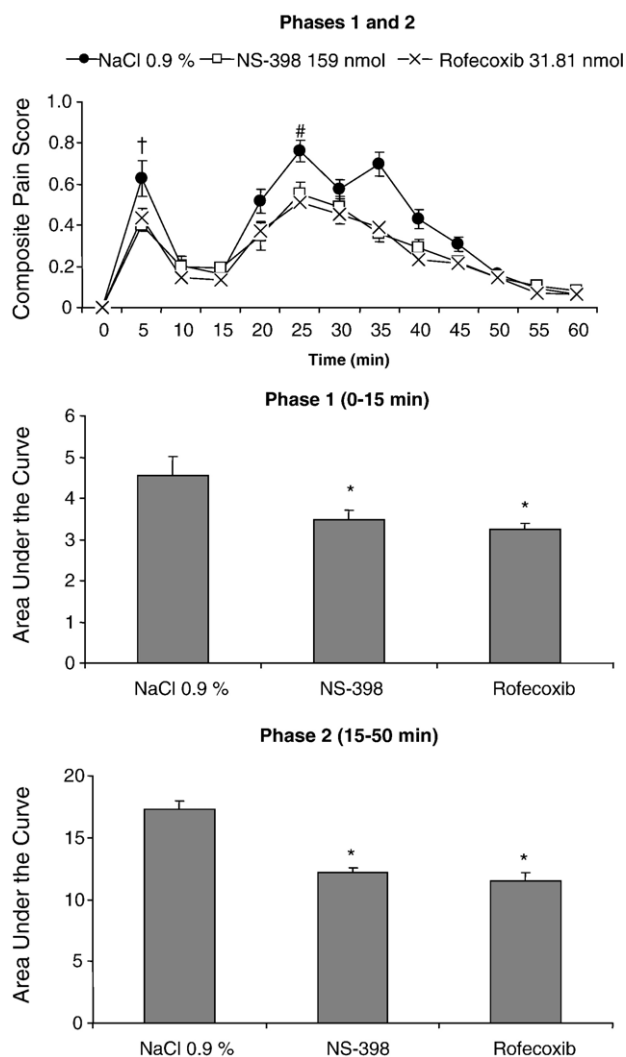

Fig. 4. Effects of NS-398 (159 nmol) and rofecoxib (31.81 nmol) given into the hind paws 15 min before 2.5% formalin (50 μl). The upper part of the figure displays the analgesic effects of the drugs compared to control. Phase 1: Analgesic effect of NS-398 and rofecoxib for the acute phase of the test (0–15 min). Phase 2: Analgesic effect of NS-398 and rofecoxib for the inflammatory phase of the test (15–50 min). Data is expressed as mean±S.E.M. ($n=7$). †Area Under the Curve (AUC) for the acute phase, $P<0.005$ for analgesics vs. NaCl 0.9%; #AUC for the inflammatory phase, $P<0.001$ for analgesics vs. NaCl 0.9%; * NS-398 or Rofecoxib vs. NaCl 0.9%, $P<0.005$.
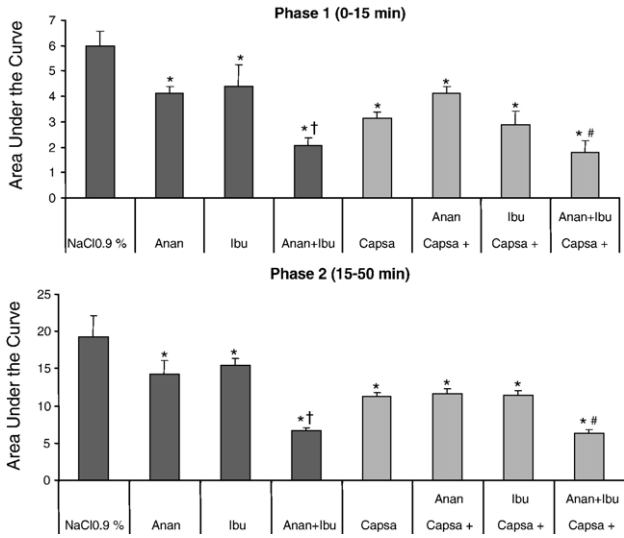
Fig. 5. Effect of capsazepine on ibuprofen, anandamide and their combination. Acute phase (0–15 min) (Phase 1). Inflammatory phase (15–50 min) (Phase 2). Data is expressed as mean±S.E.M. ($n=4$). Anan = anandamide (ED_{50} dose), Ibu=ibuprofen (ED_{50} dose), Capsa=capsazepine (2.65 μmol). * drugs vs. control group, $P<0.001$; †anan+ibu vs. anandamide, $P<0.005$; #capsa+anan+ibu vs. capsa, $P<0.01$.

with capsazepine produced a significant antinociceptive effect compared to the control group in the acute ($P<0.001$) and the inflammatory phases ($P<0.001$) (Fig. 5). The combination of anandamide with ibuprofen produced lower pain behaviour values compared to the anandamide group ($P<0.005$ and $P<0.001$ for the acute and inflammatory phases, respectively) (Fig. 5). The association of capsazepine with anandamide and ibuprofen produced lower pain behaviour values in comparison to the capsazepine group ($P<0.01$ and $P<0.005$ for the acute and inflammatory phases, respectively) (Fig. 5).
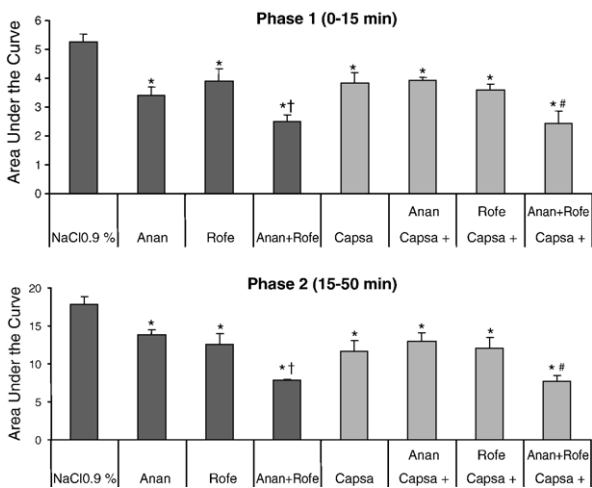

Fig. 6. Effects of capsazepine on rofecoxib, anandamide and their combination. Acute phase (0–15 min) (Phase 1). Inflammatory phase (15–50 min) (Phase 2). Data is expressed as mean±S.E.M. ($n=4$). Anan = anandamide (ED_{50} dose), Rofe = rofecoxib (ED_{50} dose), Capsa = capsazepine (2.65 μmol). * drugs vs. control group, $P<0.001$; †anan+rofe vs. anandamide, $P<0.01$; #capsa+anan+rofe vs. capsa, $P<0.005$.

3.5. TRPV1 receptors are not implicated in the antinociceptive effect of the anandamide–rofecoxib combination

Local administration of capsazepine was associated with a significant antinociceptive effect. Furthermore, when given locally at ED_{50} doses, anandamide, rofecoxib and their combination alone or in association with capsazepine produced a significant antinociceptive effect compared to the control group in the acute ($P<0.001$) and the inflammatory phases ($P<0.001$) (Fig. 6). The combination of anandamide with rofecoxib generated lower pain behaviour values compared to the anandamide group ($P<0.01$ and $P<0.001$ for the acute and inflammatory phases, respectively) (Fig. 6). The association of capsazepine with anandamide and rofecoxib produced lower pain behaviour values in comparison to the capsazepine group ($P<0.001$ and $P<0.005$ for the acute and inflammatory phases, respectively) (Fig. 6).
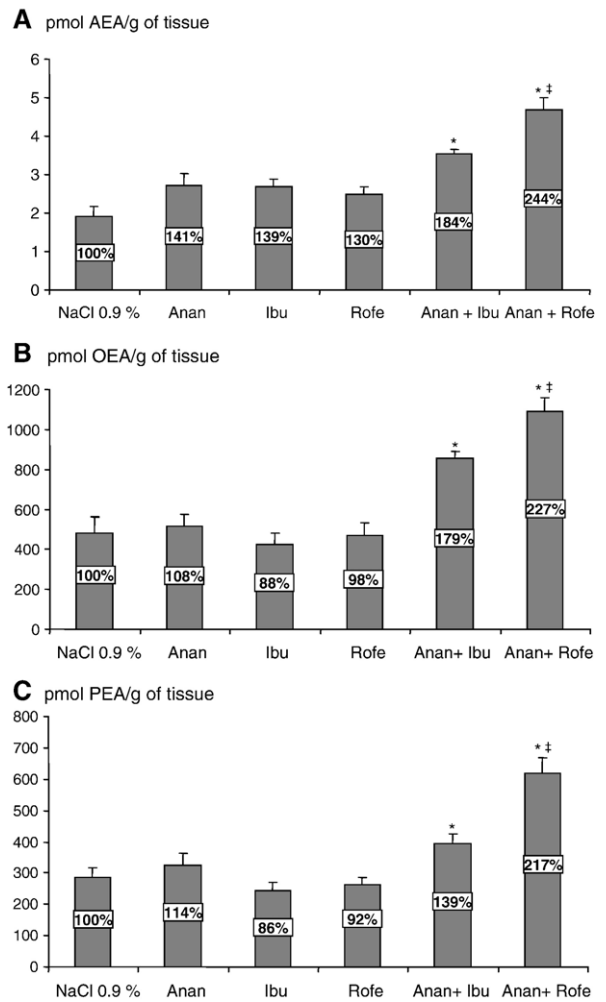
Fig. 7. Amount in pmol/g of tissue of anandamide (A), oleoylethanolamide (B) and palmitoylethanolamide (C) following local administration of anandamide, ibuprofen, rofecoxib or their combinations. Data is expressed as mean±S.E.M. ($n=4$). Anan = anandamide (ED_{50} dose), Ibu = ibuprofen (ED_{50} dose), Rofe = rofecoxib (ED_{50} dose). *anandamide+NSAIDs vs. drugs given alone, $P<0.001$; ‡anandamide+rofecoxib vs. anandamide+ibuprofen, $P<0.025$.

3.6. Higher concentrations of fatty-acid ethanolamides when anandamide is given with NSAIDs

The amount of anandamide, oleoylethanolamide and palmitoylethanolamide in paw tissues were significantly higher when anandamide was given locally with NSAIDs compared to the drugs given alone (Fig. 7A, B, C) ($P < 0.001$). The increase in the quantity of anandamide, oleoylethanolamide and palmitoylethanolamide in the paws was significantly higher when anandamide was combined with rofecoxib compared to its combination with ibuprofen (Fig. 7A, B, C) ($P < 0.005$, $P < 0.025$ and $P < 0.001$, respectively).

4. Discussion

We report that anandamide and rofecoxib displayed antinociceptive effects in the formalin test when injected in the paw of rats and that their combination was synergistic and did not involve TRPV1 receptors. These effects were locally mediated and not systemic as anandamide and rofecoxib given in the contralateral paw were not antinociceptive at doses higher than the ED_{50} doses used in the ipsilateral paw. Finally, exogenous administration of anandamide with NSAIDs (ibuprofen or rofecoxib) increased the levels of anandamide, oleoylethanolamide and palmitoylethanolamide in inflamed paw tissues.

We hypothesised that the peripheral antinociceptive effects of endocannabinoids such as anandamide are potentiated by concomitant administration with an NSAID. Indeed, the enzymatic hydrolysis of anandamide by FAAH is blocked by ibuprofen, ketorolac and flurbiprofen (Fowler et al., 1997, 1999) so that anandamide is not degraded, and thus, present locally in greater amounts to decrease pain behaviour. We have shown in a previous study that the combination of anandamide with ibuprofen produced synergistic antinociceptive effects but the mechanism of this interaction, although mediated in part by cannabinoid CB_1 and CB_2 receptors, was not clear (Guindon et al., 2006). In the present study, we have also demonstrated that the same is true for the combination of anandamide with rofecoxib and, furthermore, that it was associated with a higher quantity of anandamide and other fatty-acid ethanolamides in the paw of rats injected with formalin. There is not an obvious explanation for this finding. However, we can suggest that COX-2 is involved in the oxidative metabolism of endocannabinoids and therefore, in the presence of rofecoxib, there may be a decrease in this metabolic pathway, increasing then the local levels of fatty-acid ethanolamides compared with the use of ibuprofen. Despite the higher concentration of anandamide in paw tissues, and therefore the possibility that anandamide activated TRPV1 receptors, it is suggested that this was not the case as the interaction of anandamide with ibuprofen gave the same antinociceptive effects in the presence or absence of a TRPV1 receptor antagonist. Finally, the local antinociceptive effects of capsazepine, a TRPV1 receptor antagonist, in the two phases of the formalin test suggest the existence of a pronociceptive tone of vanilloid receptors in peripheral tissues.

4.1. Antinociceptive effects of the combination of cannabinoids with NSAIDs

The endogenous PPAR-α receptor agonist, palmitoylethanolamide (Lo Verme et al., 2005a), and indomethacin given orally have demonstrated anti-oedema and anti-inflammatory properties in a rat model of acute inflammation (Conti et al., 2002). Furthermore, Gühring et al. (2002) have shown that intrathecal indomethacin was antinociceptive in the formalin test in the rat and that this effect was antagonised by AM251, a cannabinoid CB_1 receptor antagonist. In the same study, the authors showed that intrathecal indomethacin was ineffective in cannabinoid CB_1 knockout mice. Moreover, Gühring et al. (2002) reported that pain-related behaviour in the formalin test was similar in COX wild type, $COX1^{-/-}$ and $COX2^{-/-}$ mice suggesting that COX inhibition is not the only mechanism that could explain the effects of NSAIDs. They concluded that endocannabinoids play a major role in mediating NSAIDs-induced antinociception at the spinal level. However, there is growing evidence that the antinociceptive effect of rofecoxib involves the activation of GMP-K$^+$ channel pathway (Deciga-Campos and Lopez-Munoz, 2004) and serotoninergic pathway (Sandrini et al., 2002). Recent studies suggest that diclofenac and lumiracoxib induced peripheral antinociception through the activation of the NO-cyclic GMP pathway (Lozano-Cuenca et al., 2005).

4.2. Local antinociceptive effect of cannabinoids and endocannabinoids

In this study, local administration of anandamide produced a significant reduction in pain behaviour. These findings are consistent with the ability of endocannabinoids to reduce hyperalgesia in rodent models of acute inflammation (Calignano et al., 1998; Richardson et al., 1998). Anandamide was reported to be 100 times more potent in preventing formalin-evoked pain behaviour when injected locally into the receptive field, compared to when it was administered intravenously (Calignano et al., 1998).

In the present study, anandamide was antinociceptive during the two phases of the formalin test (60 min) despite the fact that the point of its maximum inhibitory effect is usually reported to be at 10 min post-injection. The fact that anandamide was administered locally in peripheral tissue and therefore present in greater amount, as we have shown in this study, may explain its longer duration of action, whereas significant hydrolysis may account for its shortened antinociceptive effects when given systemically. Furthermore, another mechanism that would also explain the higher concentration of anandamide is the possibility of a shift of arachidonic acid metabolism towards endocannabinoid synthesis in response to COX inhibition (Gühring et al., 2002; Ates et al., 2003; Seidel et al., 2003). A third alternative, given the fact that COX-2 can metabolise anandamide *in vivo* in FAAH knock out mice (Weber et al., 2004), is that NSAIDs can also act by preventing COX-2 removal of anandamide and thereby allow its build-up. This is why rofecoxib was used to test its analgesic interaction with anandamide.

Palmitoylethanolamide has anti-inflammatory and antinociceptive properties (Calignano et al., 1998) and the anti-inflammatory effects of this compound have been shown to depend on PPAR-α receptor activation (Lo Verme et al., 2005a). In the present study, the increase in palmitoylethanolamide was greater when anandamide was given with rofecoxib compared with ibuprofen. This significant difference needs further investigation, as it suggests that rofecoxib may be able to elevate, through an as yet uncharacterised mechanism, tissue levels of this endogenous PPAR-α ligand.

4.3. Local antinociceptive effect of NSAIDs

At the site of inflammation in the periphery, inhibition of prostaglandin synthesis is a well-established mechanism by which NSAIDs exert their antinociceptive action (Taiwo and Levine, 1990). Indeed, the antinociceptive effect of NSAIDs administered locally has already been reported (Islas-Cadena et al., 1999; Aguirre-Banuelos and Granados-Soto, 2000; Francischi et al., 2002; Torres-Lopez et al., 2002; Ma and Eisenach, 2003), but the antinociceptive efficacy of selective COX-2 inhibitors compared to standard NSAIDs is controversial. In the present study, rofecoxib displayed antinociceptive effects at very low concentrations during the two phases of the formalin test but did not influence paw oedema formation. To corroborate these findings another COX-2 inhibitor, NS-398, was tested and confirmed the data obtained with rofecoxib. It is quite surprising to see that rofecoxib was analgesic in the first phase (acute) of the formalin test since COX-2 is expressed at very low levels in the periphery. However, induction of COX-2 expression may be increased by peripheral injury, leading to the development of COX-2 inhibitor sensitivity that may or may not be observed within the one hour window of the formalin test (Dirig et al., 1997). Furthermore, the same authors have already demonstrated that an acute stimulus, such as intraplantar injection of formalin, could lead to an immediate increase in prostaglandin (PG) release (Dirig et al., 1997). It is suggested that this increased PGE_2 release is mediated by COX-2 and not COX-1 pharmacology, and because the effects are observed acutely, this COX-2 must by definition be constitutively expressed (Ghilardi et al., 2004). Indeed, in many peripheral tissues COX-2 is normally expressed at very low levels but is dramatically upregulated after tissue injury (Ghilardi et al., 2004). Other authors have suggested that products of COX-2 can be released in the injected paw immediately after formalin injection and that intraplantar injection of celecoxib completely inhibited development of secondary hyperalgesia (Veiga et al., 2004). Finally, in a study by Yamamoto and Nozaki-Taguchi (2002), intrathecal administration of celecoxib and indomethacin was associated with a significant decrease in the number of flinching in both phase 1 and 2 of the formalin test compared to vehicle-treated rats, although it was not significant for the first phase when the two drugs were administered orally. Considering suggestions from Dirig et al. (1997) and Ghilardi et al. (2004) mentioned above, it is possible that intraplantar injection of NaCl 0.9% and rofecoxib or NS-398 15 min prior to injection of formalin may have induced COX-2 expression locally.

4.4. Paw tissue levels of fatty-acid ethanolamides

Other authors have already measured endocannabinoid concentrations or FAAH activity in rodent skin paw tissue (Beaulieu et al., 2000; Holt et al., 2005). However, in the study by Beaulieu et al. where levels of anandamide, palmitoylethanolamide and 2-AG were measured in rat paw skin during control and formalin-induced inflammation conditions results were expressed in pmol/mg of extracted lipids, whereas it the present study the results are expressed in pmol/g of tissues. Furthermore, we did not measure levels of endocannabinoids in control levels but after injection of saline and then formalin into the paw. Therefore direct comparisons between the two studies are difficult. However, Beaulieu et al. (2000) did not find any increase in endocannabinoid levels after the injection of formalin. FAAH activity was also not different between the two conditions. On the contrary, Holt et al. (2005) showed that FAAH activity in the paws of the inflamed vehicle-treated mice was significantly lower than the corresponding activity in the non-inflamed mice.

We have observed that there was a marked difference between the 50% increase in anandamide levels when given alone vs. anandamide + rofecoxib compared with levels of oleoylethanolamide and palmitoylethanolamide (100% increase). The explanation for such a difference is not obvious but we can suggest, at least for palmitoylethanolamide, that FAAH is also involved in its metabolism, therefore in presence of an inhibitor of FAAH activity like an NSAID, it is logical to observe such an increase. A reason could be the local pH values of the tested substances, however, the pH of ibuprofen and rofecoxib were found to be similar and not different from normal saline (data not shown). Finally, it could be that in the presence of a COX-2 inhibitor, less anandamide can be converted into its COX-2 metabolite, $PGF_{2\alpha}$ ethanolamide (Woodward et al., 2000).

One limitation of the study would be the use of DMSO in behavioural experiments. Indeed, DMSO should be avoided when possible in *in vivo* experiments due to a possible analgesic/sedative effect. However, experiments comparing saline and saline with 50% DMSO in a significant number of animals showed that no difference could be found between the two groups.

5. Conclusion

Peripheral administration of anandamide or rofecoxib is associated with a decrease in pain behaviour in animals submitted to acute and inflammatory pain. The association of an endocannabinoid with an NSAID (specific COX-2 inhibitor) produced a synergistic antinociceptive effect. This antinociceptive effect is mediated in part by cannabinoid CB_1 and CB_2 receptors (Guindon et al., 2006) but not by TRPV1 receptors. Furthermore, fatty-acid amide concentrations in paw tissues are increased when anandamide is administered with an NSAID. The contribution of the cannabinoid receptors and of other mechanisms possibly involved, such as PPAR-α receptors, needs to be further investigated.

Acknowledgments

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