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A role for endocannabinoids in indomethacin-induced spinal antinociception

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Abstract

Inhibition of prostaglandins synthesis does not completely explain non-steroidal anti-inflammatory drug-induced spinal antinociception. Among other mediators, endocannabinoids are involved in pain modulation. Indomethacin-induced antinociception, in the formalin test performed in spinally microdialysed mice, was reversed by co-administration of the cannabinoid 1 (CB_1) antagonist, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1-*H*-pyrazole-3-carboxamide (AM-251), but not by co-infusion of prostaglandin E_2. Indomethacin was ineffective in CB_1 knockout mice. AM-251 also reversed the indomethacin-induced antinociception in a test of inflammatory hyperalgesia to heat. Furthermore, during the formalin test, indomethacin lowered the levels of spinal nitric oxide (NO), which activates cellular reuptake and thus breakdown of endocannabinoids. The pronociceptive effect of an NO donor, 3-methyl-*N*-nitroso-sydnone-5-imine (RE-2047), was abolished by co-administration of the endocannabinoid transporter blocker *N*-(4-hydroxyphenyl) arachidonoyl amide (AM-404). Moreover, the antinociceptive activity of the NO synthase inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME), was reversed by AM-251. Thus we propose that at the spinal level, indomethacin induces a shift of arachidonic acid metabolism towards endocannabinoids synthesis secondary to cyclooxygenase inhibition. In addition, it lowers NO levels with subsequent higher levels of endocannabinoids.

Keywords: Spinal nociception; Formalin test; Inflammation; Heat hyperalgesia; Spinal microdialysis; Nitric oxide

1. Introduction

Even though non-steroidal anti-inflammatory drugs are widely used for their analgesic effect (Brooks, 1998), the mechanism of this effect is still puzzling. Many observations suggested that non-steroidal anti-inflammatory drugs probably act on targets other than inhibition of the cyclooxygenases, to counteract pain. For example, salicylic acid does not inhibit cyclooxygenase enzyme activity at analgesic concentrations or doses, however, as a pain relieving drug or antipyretic it is equal to acetyl salicylic acid, which itself inhibits cyclooxygenase activity at clinically relevant doses (Brune et al., 1991). At the site of inflammation in periphery, inhibition of prostaglandin synthesis is well established to be the mechanism of non-steroidal anti-inflammatory drugs-induced antinociception, e.g. (Taiwo and Levine, 1990); supplementation of exogenous prostaglandin to peripheral inflamed tissues reversed the antinociceptive effect of indomethacin in the writhing test in mice (Ueno et al., 2001), and in the acutely inflamed joints of cats (Heppelmann et al., 1986). However, at the spinal level, there is only indirect evidence that spinal prostaglandins act as pronociceptive mediators; nociceptive stimuli increase spinal prostaglandin E_2 production e.g. (Malmberg and Yaksh, 1995; Vetter et al., 2001), and intrathecal (i.t.) injection of cyclooxygenase inhibitors produces a dose dependent antinociceptive effect in the formalin test in rats (Malmberg and Yaksh, 1992a). Prostaglandin E_2 unquestionably contributes to spinally mediated allodynia (Minami

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et al., 1994), but direct evidence for pronociceptive effects of intrathecally applied prostaglandin E_2 in the formalin test—which is believed to be a more valid model for clinical pain than other threshold escape models (Tjolsen et al., 1992)—is lacking. There is no evidence in the literature that supplementing prostaglandin E_2 diminished an antinociceptive effect of a cyclooxygenase inhibitor at the spinal level. In addition, we have recently shown that at the spinal level, the synthetic cannabinoid *R*(−)-7-hydroxy-delta-6-tetrahydrcannabinol-dimethylheptyl (HU-210) induced prostaglandin E_2 synthesis simultaneous to its antinociceptive effect (Gühring et al., 2001a).

The discovery of endocannabinoids opened up new insights into nociception; their antinociceptive effects were reported in several pain models e.g. (Mechoulam et al., 1995; Pertwee, 2001). Arachidonoyl ethanolamide (anandamide) the endogenous ligand for cannabinoid CB_1 receptors was shown to be a metabolite of arachidonic acid (Pestonjamasp and Burstein, 1998). Although it is not clear so far through which pathway arachidonic acid contributes to the synthesis of anandamide or other endocannabinoids (Kuwae et al., 1999), it was shown that arachidonic acid mobilization is a condition favouring increased anandamide synthesis (Pestonjamasp and Burstein, 1998). Moreover, Berger et al. (2001) reported higher levels of biologically active *N*-acylethanolamines in the brain of piglets fed with long-chain polyunsaturated fatty acids supplemented diet. However, under physiological conditions anandamide is thought to be synthesised via a phospholipase D catalysed hydrolysis of *N*-arachidonoyl phosphatidylethanolamine (Sugiura et al., 2002).

Recent studies, performed on various cell cultures and tissues, demonstrate that the life span of endocannabinoids is limited by a rapid cellular uptake of endocannabinoids via a membrane transporter with subsequent intracellular degradation (Giuffrida et al., 2001). AM-404 selectively blocks this transporter and protects endocannabinoids from degradation in the cells, whereas nitric oxide (NO) has just an opposite effect (Bisogno et al., 2001; Maccarrone et al., 1998, 2000). If there is a tonic release of endocannabinoids, then the endocannabinoid transporter inhibitor, AM-404, should alleviate pain. But so far, this has been tested only in acute nociceptive models providing negative results (Beltramo et al., 2000).

It has been frequently speculated that cyclooxygenase inhibition results in an accumulation of arachidonic acid with subsequent diversion of its metabolism to the lipoxygenase and epoxygenase pathways, e.g. in case of aspirin-induced asthma, postulated years ago (e.g. Szczeklik et al., 1975). However, the possibility of a shift towards endocannabinoids formation was never tested. If an endocannabinoid shift occurs in response to cyclooxygenase inhibition, it might play an important role in indomethacin-induced antinociception. There is so far no evidence for a functional role of the endocannabinoid transporter in pain processing (Giuffrida et al., 2001; Pertwee, 2001), whereas the pronociceptive role of NO donors is well established (Gühring et al., 2001b; Inoue et al., 1997) without a convincing causal explanation.

In the present work, we investigated the possibility of involvement of endocannabinoids in indomethacin-induced antinociception at the spinal level.

2. Materials and methods

2.1. Experimental animals

Animals used for the study were either C57/BL6 mice weighing between 18 and 25 g bred in the Institute of Pharmacology, University of Erlangen-Nürnberg, cannabinoid CB_1 receptors wild type ($CB_1^{+/+}$) and cannabinoid CB_1 receptor knockout ($CB_1^{-/-}$) mice generated as described (Ledent et al., 1999), or cyclooxygenase wild type, cyclooxygenase 1 knockout ($COX1^{-/-}$) and cyclooxygenase 2 knockout mice ($COX2^{-/-}$) (strain numbers: 5002W, 5001M, 5002M, respectively; Taconic, Germantown, NY).

Animals were housed in groups and were allowed free access to food and water until the start of experiments.

The ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983) were followed and experiments were approved by the local ethical committee.

2.2. Materials

N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1-*H*-pyrazole-3-carboxamide (AM-251) was purchased from Alexis (Grünberg, Germany). *N*-nitrio-L-arginine methyl ester (L-NAME), indomethacin, and prostaglandin E_2, were obtained from Sigma (Deisenhofen, Germany). *N*-(4-hydroxyphenyl) arachidonoyl amide (AM-404) and arachidonoyl ethanolamide (anandamide) were purchased from Cayman Chemical (Ann Arbor, MI, USA). 3-Methyl-*N*-nitroso-sydnone-5-imine (RE-2047) (Rehse and Ciborski, 1995) was a generous gift from Prof. Rehse (Department of Pharmaceutical Chemistry, Berlin). Except for indomethacin which was dissolved in 1 M sodium bicarbonate, all drugs were dissolved in ethanol, Tween 80 was added, and then diluted in artificial cerebrospinal fluid (ACSF) or phosphate buffered saline (PBS) for intrathecal and intraperitoneal (i.p.) injection, respectively, or ACSF for perfusion through the microdialysis fibre. One percent black ink was added in case of intrathecal injection for verifying the injection site. Final concentration of ethanol and Tween 80 was less than 1% and 0.25%, respectively, in all cases.

2.3. Spinal microdialysis in the freely moving mice

The procedure was performed as described previously (Gühring et al., 2001a). In short, under isoflurane anaes-

thesia, the dialysis fibre (Cuprophan hollow fibre, outer diameter 216 μm, molecular weight cut-off 36-kDa, Hospal, Nürnberg, Germany) was introduced through the intervertebral joints between the thoracic and lumbar segments, to pass transversely through the dorsal horn. The insertion was performed blindly. The dialysis fibre was then connected on both sides to silicon tubes (inner diameter 0.3 mm, outer diameter 0.5 mm) using cyanacrylate glue. A small diameter aluminium tube was used as a holder for the thin silicon tubes to protect them from being scratched off. The silicon tube on one side was connected to a microdialysis pump (CMA 100, CMA/Microdialysis) and ACSF was perfused at a flow rate of 5 μl/min. The experimental protocol is summarized in Fig. 4C. Mice showing neurological abnormalities were excluded. Animals were allowed free access to food and water. Samples were collected for subsequent analysis of prostaglandin E_2 and NO degradation products. Proper placement of microdialysis tubes in the dorsal horn was verified by inspecting the ink filled fibres in cross sections at the thoracolumber junction.

2.4. Application of drugs

Indomethacin, AM-251 and prostaglandin E_2 were perfused through the microdialysis fibre. Direct intrathecal injection was performed as described (Hylden and Wilcox, 1980). Briefly, drugs were injected intrathecally at the thoracolumbar junction (corresponds to L5 and L6 of the spinal cord) under isoflurane anaesthesia 5 or 30 min before formalin test. The volume injected was always 3 μl.

2.5. Formalin test

The formalin test was performed as described elsewhere (Dubuisson and Dennis, 1977). Briefly, 20 μl of 5% formalin was injected subcutaneously into the dorsal surface of the hind paw. The animal behaviour was continuously monitored for the next 60 min. The pain related behaviour was calculated as the sum of the total number of flinches (rapid shaking of the injected paw). Microdialysed mice were briefly anaesthetised for few seconds with isoflurane to facilitate their restraining during formalin injection without disconnection of the dialysing tubes. Formalin was injected after complete recovery from anaesthesia.

2.6. Zymosan-induced heat hyperalgesia

The procedure was performed as previously described (Gühring et al., 2000). Briefly 20 μl of zymosan (3 mg/ml suspension) were injected subcutaneously into the plantar side of one hind paw. Paw withdrawal latencies were determined on exposure of the paws to a defined radiant heat stimulus using a commercially available apparatus (Hargreaves Test, UGO BASILE Biological Research Apparatus, Comerio, Italy).

2.7. Determination of spinal prostaglandin E_2 concentration

Aliquots (15 μl) of the dialysate samples were incubated with 75 μl enzyme immunoassay buffer for prostaglandin E_2 measurements. All further steps were performed as described in the Cayman Chemical prostaglandin E_2 enzyme immunoassay kit-monoclonal, calibration range: 1000–7.8 pg/ml (Cayman Chemicals). Measurements were completed using an enzyme-linked immunosorbent assay reader (DIAS Microplate reader, Dynatech Laboratories, Great Britain) with an absorbance maximum at 405 nm.

2.8. Determination of spinal NO degradation products

For indirect NO measurement a sensitive fluorescent nitrite assay was used as described previously (Misko et al., 1993). Ten microliters of a freshly prepared solution of 0.05 mg/ml 2,3-diaminonaphthalene (Sigma) in 0.62 M HCl were added to 50 μl of dialysate fluid and incubated in the dark for 10 min. The reaction was terminated by addition of 5 μl of 2.8 M NaOH and the fluorescence was read immediately at 355/460 nm in a Fluoroskan Ascent FL 2.2 (MTX Lab Systems). The standards consisted of freshly prepared sodium nitrite (4000–31.25 nM) dissolved in ACSF.

2.9. Data analysis

Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Dunnett or Bonferroni post-hoc tests ($P<0.05$ was considered statistically significant). For comparing $CB_1^{+/+}$ and $CB_1^{-/-}$ mice, Student's *t*-test was used. All results are expressed as means ± S.E.M.

3. Results

3.1. Prostaglandin E_2 substitution does not reverse the antinociceptive activity of indomethacin

As previously observed, subcutaneous injection of formalin into the hind paw of mice resulted in two phases of pain behaviour, mainly flinching and licking of the injected paw. Results from microdialysed mice were not obviously different from those obtained from non-operated mice. To test the widely assumed pronociceptive role of spinal prostaglandin E_2, we perfused it through the spinal microdialysis fibre alone and in combination with indomethacin. The choice of concentrations of prostaglandin E_2 (568 nM and 5.68 μM) was based on a pilot study, in which two spinal microdialysis fibres were inserted one bone segment apart; one was perfused with prostaglandin E_2 and the second with vehicle. The dialysate from the latter was collected and its prostaglandin E_2 content was measured.

In case of the higher concentration, the spinal prostaglandin E_2 level was at least threefold higher than the last sample before drug infusion started. Unexpectedly, both doses of prostaglandin E_2 used lowered the behavioural response to formalin, although not significantly (Fig. 1A).

Indomethacin (9 μM), perfused through the microdialysis probe of spinally microdialysed freely moving mice, had as expected a significant antinociceptive effect on the formalin-induced pain related behaviour. The substitution of prostaglandin E_2 for restoring its spinal levels did not reverse the antinociceptive activity of indomethacin in the microdialysed mice (Fig. 1A). The advantage of testing this in microdialysed animals was the continuous infusion of the substance, thus avoiding the possible breakdown of prostaglandin E_2 reported to occur over 50 min (Minami et al., 1994) thus, providing the opportunity to work under steady state conditions.

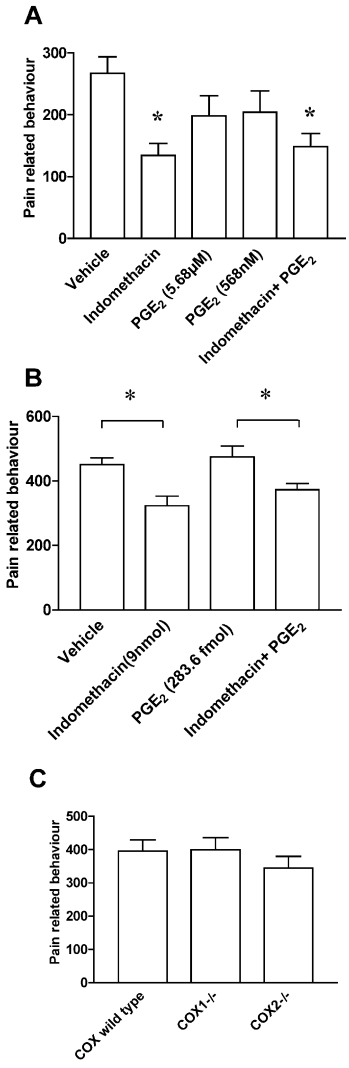

Fig. 1. Prostaglandin E_2 substitution does not reverse the antinociceptive activity of indomethacin in the formalin test when co-injected. (A) Effect of vehicle, indomethacin (9 μM), prostaglandin E_2 (PGE_2; 285 nM) or prostaglandin E_2 (PGE_2; 5.68 μM) alone or together with indomethacin, perfused through the spinal microdialysis fibre, on the formalin-induced pain related behaviour ($n=8$ except for the control group = 16). (B) Effect of vehicle, indomethacin (9 nmol), prostaglandin E_2 (PGE_2; 283.6 fmol) or both together, injected intrathecally 30 min before the formalin test, on pain related behaviour of C57/BL6 mice ($n=6-8$ animals for each group). (C) Pain related behaviour in response to subcutaneous formalin in cyclooxygenase wild type, $COX1^{-/-}$ and $COX2^{-/-}$ mice ($n=9-11$ animals for each group). Asterisk indicates significant difference compared to the vehicle group unless otherwise specified.

It was previously reported that chronically implanted intrathecal cannulae could result in nociceptive spinal sensitisation to mechanical, chemical and heat stimulation due to an ongoing mild inflammation (Almeida et al., 2000). To exclude any possible effects of an ongoing inflammation in the spinal cord due to the microdialysis operation, the failure of prostaglandin E_2 to reverse indomethacin-induced antinociception in the formalin test was confirmed using direct intrathecal injections 30 min before the beginning of the formalin test. The choice of prostaglandin E_2 dose was according to an earlier report in which prostaglandin E_2, injected intrathecally, induced heat hyperalgesia over a wide range of doses; between 50 pg/kg and 500 ng/kg (Minami et al., 1994). Since 500 ng/kg lowered itself the behavioural response to formalin (data not shown), 5 ng/kg (283.6 fmol) was used, which resulted in a nociceptive response to formalin comparable to that of the control group. Indomethacin (9 nmol, i.t.) produced antinociception when compared to the control group. The co-administration of indomethacin together with prostaglandin E_2 produced also a significant antinociception when compared to the prostaglandin E_2 injected group (Fig. 1B).

If prostaglandins play a major role in the formalin induced nociception at the spinal level, then cyclooxygenase knock out mice would be expected to show a lower nociceptive response to formalin, particularly in the second phase, which is significantly suppressed by intrathecal application of NSAIDs (Malmberg and Yaksh, 1992a). However, neither cyclooxygenase 1 nor cyclooxygenase 2 knockout mice showed phenotypic differences in response to formalin compared to the wild type mice (Fig. 1C).

These results indicate that prostaglandin E_2 neither plays a pivotal role in mediating formalin-induced nociception nor does lowering its level represent a main mechanism for indomethacin-induced antinociception in this pain model.

3.2. Indomethacin decreases spinal NO production

Since NO is known to enhance nociception at the spinal level, and several non-steroidal anti-inflammatory drugs were shown to modulate NO production and to enhance the expression of the inducible nitric oxide synthase enzyme both at the m-RNA level and at the protein level (e.g. Hinz et al., 2001), the effect of indomethacin on spinal NO production was investigated. Perfusion of indomethacin (9 μM) in the microdialysis fibre lowered spinal NO production during the formalin test. Also, prostaglandin E_2 (5.68

μM; perfused in the microdialysis fibre) showed this inhibitory effect (Fig. 2).

3.3. NO mediates inactivation of endocannabinoids

Intrathecally applied anandamide (3 nmol; 5 min before the beginning of the formalin test) reduced pain related behaviour. This effect was especially pronounced during the first 35 min after formalin injection. AM-251 (3 nmol), administered intrathecally 30 min prior to anandamide, completely abolished the effect of the latter (Fig. 3A), indicating the role of cannabinoid CB_1 receptors in anandamide-induced antinociception in the formalin test.

The endocannabinoid reuptake blocker AM-404 (3 nmol, i.t.) reduced pain related behaviour nearly as effectively as anandamide. Moreover, simultaneous administration of anandamide (i.t.) did not augment this effect (Fig. 3B), suggesting that endocannabinoids released in response to formalin injection, are alone able to produce the observed antinociception, when they are prevented from their natural rapid degradation. However, the failure of exogenous anandamide to augment the antinociceptive effect of AM-404 might also be due to activation of vanilloid VR_1 receptors by AM-404, in analogy to the preventive effect of AM-404 on anandamide-induced vasorelaxation, which was previously reported (Zygmunt et al., 2000).

The metabolic NO-donor RE-2047 (45 mg/kg, i.p., 30 min before formalin test) produced the expected increase in nociceptive response to formalin (Shibuta et al., 1996). This effect was considerably reduced, but not fully reversed, when anandamide was intrathecally injected 5 min before the test began. The combination of anandamide with AM-404, as well as AM-404 alone, completely blocked the pronociceptive effect of RE-2047. Furthermore they brought the nociceptive response down to the level of anandamide treated animals, indicating complete disruption of the pronociceptive input of NO (Fig. 3B).

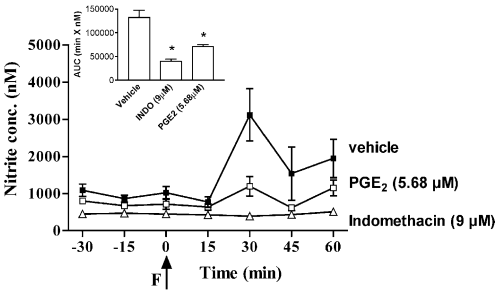

Fig. 2. Indomethacin decreases spinal NO production. Microdialysis study of spinal NO production during the formalin test in control animals, infused with indomethacin (9 μM), or prostaglandin E_2 (PGE_2; 5.68 μM). "F" points to the time of formalin injection. Areas under the time concentration curves between −30 and 60 min are presented above ($n=8$ animals in each group). Asterisk indicates significant difference compared to the vehicle group.

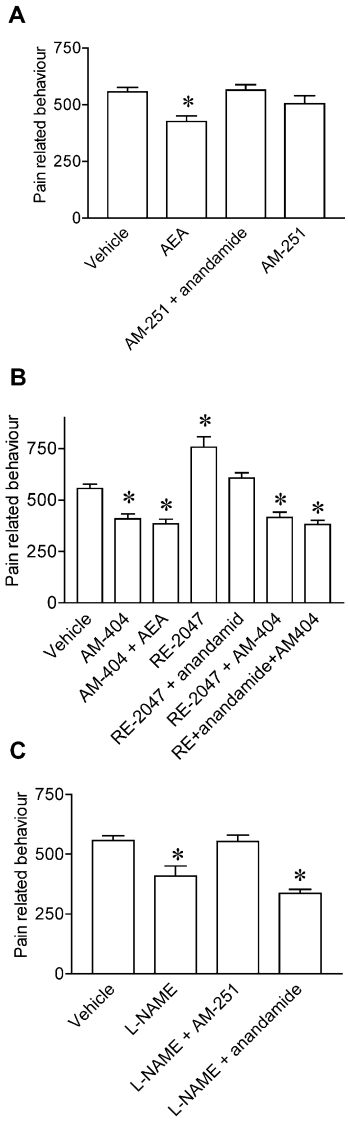

Fig. 3. Cannabinoid CB_1 receptor is involved in anandamide and L-NAME-induced antinociception. (A) Effect of vehicle, anandamide (3 nmol), AM-251 (3 nmol), or both together on the formalin-induced pain related behaviour. (B) Effect of vehicle, AM-404 (3 nmol), RE-2047 (45 mg/kg), and different combinations with anandamide (3 nmol), on the formalin-induced pain related behaviour. (C) Effect of vehicle, L-NAME (3 nmol), L-NAME together with AM-251 (3 nmol each), or L-NAME together with anandamide (3 nmol each), on the formalin-induced pain related behaviour. Asterisk indicates significant difference compared to the vehicle group. All drugs were injected intrathecally 5 min before formalin test except AM-251 and RE-2047 or its vehicle (PBS), which were injected 30 min before the formalin test intrathecally or intraperitoneally, respectively. All animals received an intraperitoneal injection and an intrathecal injection ($n=6-8$ animals for each group).

The NO synthase inhibitor L-NAME (3 nmol, i.t.) decreased the nociceptive response to formalin as expected (Malmberg and Yaksh, 1993). This effect was completely abolished by co-administration of AM-251 (Fig. 3C), indicating the involvement of endocannabinoids in L-NAME-induced antinociception at the spinal level.

3.4. Cannabinoid CB_1 receptors are involved in the antinociceptive activity of indomethacin

To investigate the possibility of an indomethacin-induced "endocannabinoid shift" as a component of its analgesic activity, we tested the effect of co-administration of a cannabinoid CB_1 receptor antagonist on the antinociceptive activity of indomethacin in two different animal pain models. Concomitant perfusion of the selective cannabinoid CB_1 receptor antagonist, AM-251 (9 μM) in the microdialysis fibre, reversed the antinociceptive activity of indomethacin (9 μM) in the formalin test (Fig. 4A). Such reversal of the antinociceptive effect of indomethacin was also observed in a second model; the zymosan induced heat hyperalgesia (Gühring et al., 2000; Hargreaves et al., 1988). Intrathecally applied indomethacin (9 nmol), inhibited zymosan induced heat hyperalgesia over 8 h, but this inhibitory effect was abolished when AM-251 (3 nmol, i.t.) was co-administered (Fig. 4B).

As previously shown (Gühring et al., 2001a), AM-251 increased spinal prostaglandin E_2 production. However, it did not modulate the inhibitory effect of indomethacin on PGE_2 production, since co-infusion of AM-251 and indomethacin revealed similar reduction in prostaglandin E_2 release as indomethacin alone (Fig. 4C).

Knockout mice deficient in the cannabinoid CB_1 receptor ($CB_1^{-/-}$) represent a useful tool for studying the involvement of endocannabinoids in indomethacin-induced antinociception. In $CB_1^{-/-}$ mice, the formalin response was more expressed compared to $CB_1^{+/+}$mice ($P<0.0001$, unpaired two-tailed Student's *t*-test), indicating the importance of the endocannabinoid tone in the mouse formalin test (Fig. 4D). Indomethacin (9 nmol, i.t., 30 min before formalin test) was ineffective in both $CB_1^{+/+}$ and $CB_1^{-/-}$ mice. However, a higher dose of indomethacin (30 nmol) was effective in $CB_1^{+/+}$, but not in $CB_1^{-/-}$ mice, emphasising the role of cannabinoid CB_1 receptors in indomethacin-induced analgesia (Fig. 4D).

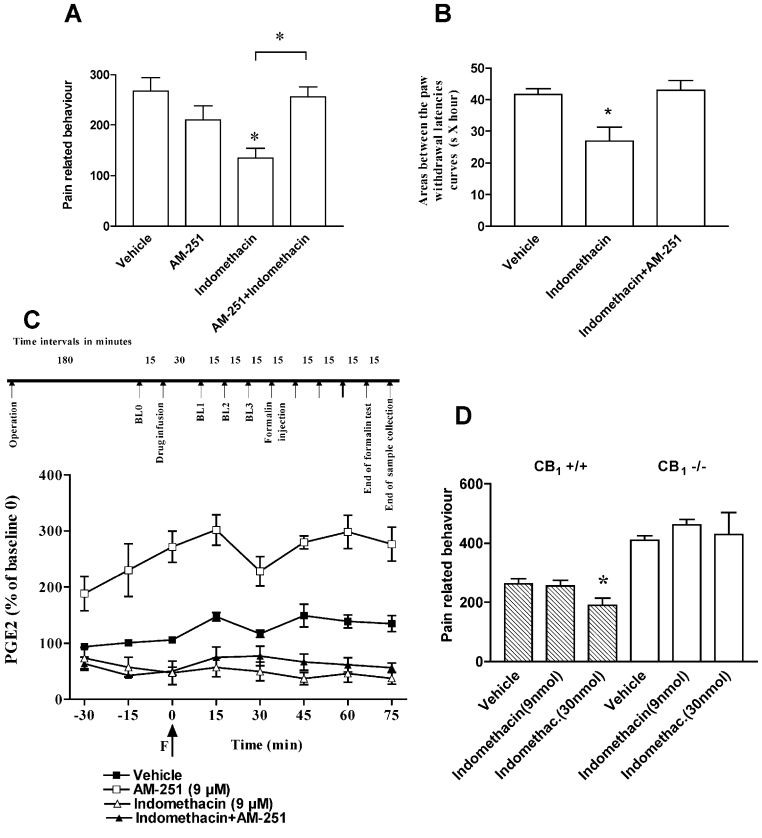

Fig. 4. AM-251 reverses the antinociceptive activity of indomethacin. (A) Effect of vehicle, indomethacin (9 μM), AM-251 (9 μM) or both together, infused in spinally microdialysed mice on the formalin-induced pain related behaviour. Asterisk indicates significant difference compared to the vehicle group and to the AM-251+ indomethacin group ($n=8$ except the control group = 16). (B) Zymosan induced heat hyperalgesia; effect of vehicle, indomethacin (9 nmol) alone or together with AM-251 (3 nmol), injected intrathecally 30 min before zymosan on hyperalgesia. The withdrawal latencies of both zymosan injected and non-injected hind paws were determined for each mouse at each time point. The graph represents areas between the paw withdrawal latencies curves of zymosan injected and non-injected mice hind paws ($n=7-11$ animals for each group). (C) Microdialysis study of spinal prostaglandin E_2 production during the formalin test in control animals, infused with indomethacin (9 μM), AM-251 (9 μM) or both together. Values are presented as percentage of baseline 0 (BL 0; last sample before drug perfusion started). "F" points to the time of formalin injection. An overview of the experimental protocol of microdialysis experiments is shown above ($n=8$ except for the control group = 16). (D) Effect of vehicle, indomethacin (9 nmol), or indomethacin (30 nmol), injected intrathecally 30 min before the formalin test on pain related behaviour of $CB_1^{+/+}$ and $CB_1^{-/-}$ mice. Asterisk indicates significant difference compared to the vehicle group.

Together, these results indicate the involvement of the endocannabinoid system in indomethacin-induced antinociception.

4. Discussion

It is known that in the presence of a continuous nociceptive afferent barrage into the dorsal horn there is a progressive generation of an arachidonic acid pool available for conversion (Malmberg and Yaksh, 1992b). In the formalin test, but not in the hot plate or tail flick tests, the stimulus-evoked activation of the arachidonic acid cascade can influence the behavioural response (Yaksh and Malmberg, 1994); e.g. it was reported that anandamide (1 fmol, i.t.) completely blocked carrageenan-induced heat hyperalgesia. However, at doses as high as 100 pmol anadamide had no effect on heat withdrawal latencies in naive (non-inflamed) animals (Richardson et al., 1998). For this reason, the formalin test and zymosan induced hyperalgesia models were chosen for this study. Performing the formalin test in microdialysed freely moving mice serves to investigate the changes in spinal production of prostaglandin and NO during the test and to correlate these changes to the behavioural responses to formalin (Malmberg and Yaksh, 1995). Indomethacin was chosen for this study as a non-selective cyclooxygenase inhibitor. It is among the most potent inhibitors of prostaglandins synthesis (Ferrari et al., 1990).

One important finding of the present work is that prostaglandin E_2 in the tested concentrations (spinal microdialysis) or doses (intrathecally applied), induced antinociception rather than pronociception in the formalin test (Fig. 1A). Although this result is contradictory to the pronociceptive role of prostaglandin E_2 reported in the literature, yet, spinal prostaglandin E_2 administration was never tested before for its effect on the formalin-induced nociception. Moreover, this finding is inline with the observation that prostaglandin E_2 was able to reduce spinal NO production, which may lower the hyperalgesic effect of PGE_2. There are reports in the literature of a putative cross talk between NO synthase and cyclooxygenase (for review, see Weinberg, 2000). For example, prostaglandin E_2 was shown to repress the induction of iNOS in mouse macrophages (Marotta et al., 1992; Milano et al., 1995; Pang and Hoult, 1997). On the other hand NO was shown to inhibit prostaglandin production in J774 mouse macrophages (Swierkosz et al., 1995) and in rat peritoneal macrophages (Habib et al., 1997), and to enhance prostaglandin biosynthesis in various cell lines, e.g. (Salvemini et al., 1995). However, the mechanism of a direct and fast interaction between them is unclear so far. On the other hand, the lower level of NO production after prostaglandin E_2 perfusion may be an unspecific wash out effect due to an increased blood flow within the spinal cord. The failure of prostaglandin E_2 substitution to reverse the antinociceptive effect of indomethacin argues against an important role for prostaglandins E_2 in its mechanism of antinociception at the spinal level. In this context it is worthwhile to mention that neither the prostaglandin receptor EP_1 nor EP_3 knockout mice showed differences in the formalin test compared to their wild types (Minami et al., 2001). The same lack of phenotype was observed here for the cyclooxygenase knockout mice. Taken together, these findings lower the possibility for the involvement of the cyclooxygenase prostanoids pathway in formalin-induced nociception at the spinal level, despite of the effectiveness of non-steroidal anti-inflammatory drugs as mentioned above.

Since the cannabinoid CB_1 receptors represent the predominate isoform within the central nervous system (for review, see Howlett et al., 2002), our study was carried out using either AM-251 or $CB_1^{-/-}$ mice. It was reported that in some cases, the cannabinoid CB_1 receptor antagonist SR141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxyamide hydrochloride) was less potent against anandamide-induced antinociception than against non-eicosanoid CB_1 receptor agonists; e.g. the ED_{50} of SR141716A for antagonism of intrathecal anandamide in the mouse tail flick test (15.4 mg/kg, i.p.) is greater than that for antagonism of intrathecal delta 9-tetrahydrocannabinol (0.76), delta 8-tetrahydrocannabinol (0.9), (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol (CP55940; 0.1) or deoxy-HU-210 (0.4; Welch et al., 1995). In other cases SR141716A even failed to antagonize anandamide altogether, e.g. SR141716A did not antagonise anandamide neither in the mouse tail flick test when both were intravenously administered (Adams et al., 1998), nor the rat paw pressure test when both were administered intraperitoneally (Smith et al., 1998). However, none of those reports involved the formalin pain model, and the present results with both anandamide and AM-251, which is a more selective cannabinoid CB_1 receptor antagonist (Gatley et al., 1996; White et al., 2001), clearly show that anandamide-induced antinociception in the formalin test is mediated via cannabinoid CB_1 receptors. In agreement with our results, SR141716A was reported to reverse anandamide-induced antinociception in the tail immersion test in fatty acid amidohydrolase (FAAH) knockout mice (Cravatt et al., 2001), indicating that the rapid degradation of anandamide might account for such differences among different studies. Such differences might also be explained by the suggestion that cannabinoid-induced antinociception in different pain models might be mediated by different cannabinoid receptor types/subtypes (Pertwee, 2001).

$CB_1^{-/-}$ mice were reported to show less pain response in the formalin test than wild type controls (Zimmer et al., 1999). In contrast, in our study $CB_1^{-/-}$ mice showed higher pain response to formalin compared to the wild type mice. This contradiction might be due to the different backgrounds of both strains and/or the different observation time

for the formalin response, which was only between 1–6 and 15–20 min in the latter report of Zimmer et al. It is important to note that results of spontaneous activity and hot plate tests were also different in both CB_1 knockout strains (for comparison, see Howlett et al., 2002; Iversen, 1999). The strain difference between the $CB_1^{+/+}$ (CD1 background strain) and C57/BL6 used in other experiments of this study may also account for the non-responsiveness to indomethacin except at a higher dose, especially with the relatively low pain response in the $CB_1^{+/+}$ used. However, our result of a higher nociceptive response to formalin in $CB_1^{-/-}$ mice indicates a tonic modulatory role of the endocannabinoid system in the formalin pain model, which is in line with the antinociceptive effect of the endocannabinoid transporter inhibitor, AM-404 in the same test reported here. The non-responsiveness of $CB_1^{-/-}$ mice to indomethacin and the reversal of its effect in wild type mice by AM251 indicate the involvement of the endocannabinoid system in its antinociceptive effect. In order to reproduce our findings in a different species, similar unpublished results have been obtained with flurbiprofen and AM-251 in rats (Mehmet Ates, May Hamza, Kay Seidel, Carolin E. Kotalla, Hans Gühring, in preparation).

As already mentioned, it is unlikely under physiological condition that anandamide is synthesised by direct condensation of arachidonic acid and ethanolamine through the action of anandamide synthase enzyme (Sugiura et al., 2002). However, the presence of a continuous nociceptive afferent input to the dorsal horn might activate this pathway. So far no "anandamide synthase" has been identified except the fatty acid amidohydrolase acting in reverse (Fig. 5). However, it requires very high concentrations of substrates to synthesise anandamide (Di Marzo, 1999). Thus raising some nonresolved questions in the field of endocannabinoid research like: Can one enzyme be responsible for both the synthesis and the breakdown of anandamide? Why does anandamide level increase after inhibition of the fatty acid amidohydrolase? Can subcellular differences in distribution explain this phenomenon? Through which pathway is arachidonic acid transformed into anandamide or other endocannabinoids is beyond the scope of the present work. However, our results show clearly the involvement of endocannabinoids in the indomethacin-induced antinociception. In this regard, Pestonjamasp and Burstein (1998) reported that indomethacin induced anandamide synthesis in the presence of calcium ionophore, proposing two possible explanations for that: the protection of anandamide from its metabolism into prostaglandin ethanolamine and the shunting of free arachidonic acid away from prostaglandins synthesis.

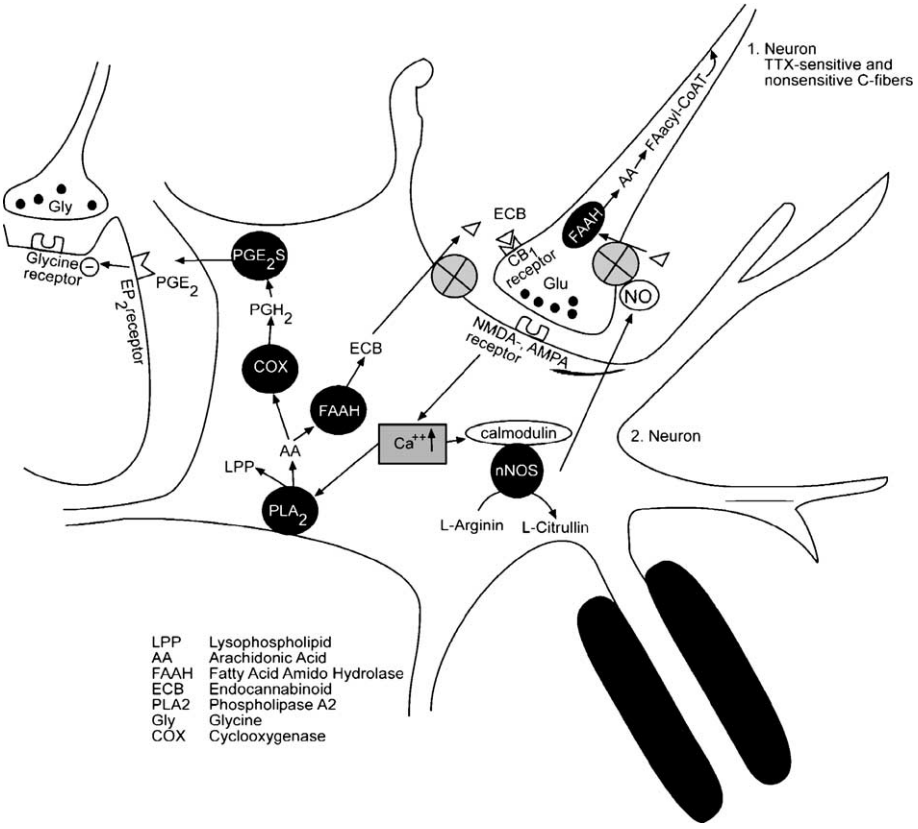

Fig. 5. Dorsal horn checkpoint of peripherally evoked pain. Indomethacin acts at least on three sites. First, it blocks the cyclooxygenase, which results in higher levels of arachidonic acid that is used for endocannabinoids synthesis "endocannabinoid shift". Second, indomethacin inhibits fatty acid amidohydrolase and prevents anandamide breakdown. Third, indomethacin lowers NO production so that it does not activate the endocannabinoids transporter.

Even though AM-404 was ineffective in the hot plate test (Beltramo et al., 2000), it showed here a potent analgesic activity in the formalin test. This might be due to the above-mentioned fact that nociceptive stimuli in the formalin test, but not in the hot plate test, activate the arachidonic acid cascade, with possible influence on the behavioural response. Our result might be a further step towards answering the question raised by several investigators (e.g. Giuffrida et al., 2001; Walker et al., 1999), whether compounds like AM-404 represent a basis for the development of a novel group of analgesics, which utilises the organism's own resources. The results of our experiments with AM-404 and RE-2047 (the metabolic NO-donor), as well as with L-NAME and AM-251, are perfectly in line with recent evidence obtained from cell cultures (Bisogno et al., 2001; Maccarrone et al., 2000), that NO activates the endocannabinoid transporter with subsequent cellular uptake and degradation of endocannabinoids. Our data support the validity of this idea in a clinically relevant in-vivo model. Consequently, by lowering the NO production, indomethacin also inhibits cellular reuptake and therefore the breakdown of endocannabinoids. The decreased NO production by indomethacin was previously shown in cell culture studies, e.g. (Hrabak et al., 2001). The inhibition of endocannabinoids breakdown by indomethacin is also achieved through inhibition of FAAH (Fowler et al., 2000).

Bradbury (2001) has recently reported a suggestion of Cravatt et al. from Scripps Research Institute, La Jolla, CA, USA. "If in-vivo fatty acid amidohydrolase inhibitors can be developed, they may provide a selective way to use the cannabinoid signalling system for chronic pain relief". The present work shows that the cannabinoid signalling system has already been used for the management of chronic pain for many years.

In conclusion, the present work adds further evidence that indomethacin may act at the spinal level at least on three sites (Fig. 5). First, it blocks the cyclooxygenases, which results in higher levels of arachidonic acid that is used for endocannabinoids synthesis "endocannabinoid shift". Second, it lowers NO production, reducing the activation of endocannabinoid transporter and hence the breakdown of endocannabinoids. Third, indomethacin inhibits FAAH, thus contributing to the sparing of endocannabinoids.

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References

Adams, I.B., Compton, D.R., Martin, B.R., 1998. Assessment of anandamide interaction with the cannabinoid brain receptor: SR 141716A antagonism studies in mice and autoradiographic analysis of receptor binding in rat brain. J. Pharmacol. Exp. Ther. 284, 1209–1217.

Almeida, F.R., Schivo, I.R., Lorenzetti, B.B., Ferreira, S.H., 2000. Chronic intrathecal cannulation enhances nociceptive responses in rats. Braz. J. Med. Biol. Res. 33, 949–956.

Beltramo, M., de Fonseca, F.R., Navarro, M., Calignano, A., Gorriti, M.A., Grammatikopoulos, G., Sadile, A.G., Giuffrida, A., Piomelli, D., 2000. Reversal of dopamine D(2) receptor responses by an anandamide transport inhibitor. J. Neurosci. 20, 3401–3407.

Berger, A., Crozier, G., Bisogno, T., Cavaliere, P., Innis, S., Di Marzo, V., 2001. Anandamide and diet: inclusion of dietary arachidonate and docosahexaenoate leads to increased brain levels of the corresponding *N*-acylethanolamines in piglets. Proc. Natl. Acad. Sci. U. S. A. 98, 6402–6406.

Bisogno, T., MacCarrone, M., De Petrocellis, L., Jarrahian, A., Finazzi-Agro, A., Hillard, C., Di Marzo, V., 2001. The uptake by cells of 2-arachidonoylglycerol, an endogenous agonist of cannabinoid receptors. Eur. J. Biochem. 268, 1982–1989.

Bradbury, J., 2001. Small part of cannabis puzzle solved by animal studies. The Lancet 358, 302.

Brooks, P., 1998. Use and benefits of nonsteroidal anti-inflammatory drugs. Am. J. Med. 104, 9S–13S (Discussion 21S–22S).

Brune, K., Beck, W.S., Geisslinger, G., Menzel-Soglowek, S., Peskar, B.M., Peskar, B.A., 1991. Aspirin-like drugs may block pain independently of prostaglandin synthesis inhibition. Experientia 47, 257–261.

Cravatt, B.F., Demarest, K., Patricelli, M.P., Bracey, M.H., Giang, D.K., Martin, B.R., Lichtman, A.H., 2001. Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. Proc. Natl. Acad. Sci. U. S. A. 98, 9371–9376.

Di Marzo, V., 1999. Biosynthesis and inactivation of endocannabinoids: relevance to their proposed role as neuromodulators. Life Sci. 65, 645–655.

Dubuisson, D., Dennis, S.G., 1977. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. Pain 4, 161–174.

Ferrari, R.A., Ward, S.J., Zobre, C.M., Van Liew, D.K., Perrone, M.H., Connell, M.J., Haubrich, D.R., 1990. Estimation of the in vivo effect of cyclooxygenase inhibitors on prostaglandin E_2 levels in mouse brain. Eur. J. Pharmacol. 179, 25–34.

Fowler, C.J., Borjesson, M., Tiger, G., 2000. Differences in the pharmacological properties of rat and chicken brain fatty acid amidohydrolase. Br. J. Pharmacol. 131, 498–504.

Gatley, S.J., Gifford, A.N., Volkow, N.D., Lan, R., Makriyannis, A., 1996. 123I-labeled AM251: a radioiodinated ligand which binds in vivo to mouse brain cannabinoid CB1 receptors. Eur. J. Pharmacol. 307, 331–338.

Giuffrida, A., Beltramo, M., Piomelli, D., 2001. Mechanisms of endocannabinoid inactivation: biochemistry and pharmacology. J. Pharmacol. Exp. Ther. 298, 7–14.

Gühring, H., Gorig, M., Ates, M., Coste, O., Zeilhofer, H.U., Pahl, A., Rehse, K., Brune, K., 2000. Suppressed injury-induced rise in spinal prostaglandin E_2 production and reduced early thermal hyperalgesia in iNOS-deficient mice. J. Neurosci. 20, 6714–6720.

Gühring, H., Schuster, J., Hamza, M., Ates, M., Kotalla, C.E., Brune, K., 2001a. HU-210 shows higher efficacy and potency than morphine after intrathecal administration in the mouse formalin test. Eur. J. Pharmacol. 429, 127–134.

Gühring, H., Tegeder, I., Lotsch, J., Pahl, A., Werner, U., Reeh, P.W., Rehse, K., Brune, K., Geisslinger, G., 2001b. Role of nitric oxide in zymosan induced paw inflammation and thermal hyperalgesia. Inflamm. Res. 50, 83–88.

Habib, A., Bernard, C., Lebret, M., Creminon, C., Esposito, B., Tedgui, A.,

Maclouf, J., 1997. Regulation of the expression of cyclooxygenase-2 by nitric oxide in rat peritoneal macrophages. J. Immunol. 158, 3845–3851.

Hargreaves, K., Dubner, R., Brown, F., Flores, C., Joris, J., 1988. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 32, 77–88.

Heppelmann, B., Pfeffer, A., Schaible, H.G., Schmidt, R.F., 1986. Effects of acetylsalicylic acid and indomethacin on single groups III and IV sensory units from acutely inflamed joints. Pain 26, 337–351.

Hinz, B., Brune, K., Rau, T., Pahl, A., 2001. Flurbiprofen enantiomers inhibit inducible nitric oxide synthase expression in RAW 264.7 macrophages. Pharm. Res. 18, 151–156.

Howlett, A.C., Barth, F., Bonner, T.I., Cabral, G., Casellas, P., Devane, W.A., Felder, C.C., Herkenham, M., Mackie, K., Martin, B.R., Mechoulam, R., Pertwee, R.G., 2002. International Union of Pharmacology. XXVII. Classification of Cannabinoid Receptors. Pharmacol. Rev. 54, 161–202.

Hrabak, A., Vercruysse, V., Kahan, I.L., Vray, B., 2001. Indomethacin prevents the induction of inducible nitric oxide synthase in murine peritoneal macrophages and decreases their nitric oxide production. Life Sci. 68, 1923–1930.

Hylden, J.L., Wilcox, G.L., 1980. Intrathecal morphine in mice: a new technique. Eur. J. Pharmacol. 67, 313–316.

Inoue, T., Mashimo, T., Shibuta, S., Yoshiya, I., 1997. Intrathecal administration of a new nitric oxide donor, NOC-18, produces acute thermal hyperalgesia in the rat. J. Neurol. Sci. 153, 1–7.

Iversen, L., 1999. High times for cannabis research. Proc. Natl. Acad. Sci. U. S. A. 96, 5338–5339.

Kuwae, T., Shiota, Y., Schmid, P.C., Krebsbach, R., Schmid, H.H., 1999. Biosynthesis and turnover of anandamide and other *N*-acylethanolamines in peritoneal macrophages. FEBS Lett. 459, 123–127.

Ledent, C., Valverde, O., Cossu, G., Petitet, F., Aubert, J.F., Beslot, F., Bohme, G.A., Imperato, A., Pedrazzini, T., Roques, B.P., Vassart, G., Fratta, W., Parmentier, M., 1999. Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. Science 283, 401–404.

Maccarrone, M., van der Stelt, M., Rossi, A., Veldink, G.A., Vliegenthart, J.F., Agro, A.F., 1998. Anandamide hydrolysis by human cells in culture and brain. J. Biol. Chem. 273, 32332–32339.

Maccarrone, M., Bari, M., Lorenzon, T., Bisogno, T., Di Marzo, V., Finazzi-Agro, A., 2000. Anandamide uptake by human endothelial cells and its regulation by nitric oxide. J. Biol. Chem. 275, 13484–13492.

Malmberg, A.B., Yaksh, T.L., 1992a. Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat. J. Pharmacol. Exp. Ther. 263, 136–146.

Malmberg, A.B., Yaksh, T.L., 1992b. Hyperalgesia mediated by spinal glutamate or substance P receptor blocked by spinal cyclooxygenase inhibition. Science 257, 1276–1279.

Malmberg, A.B., Yaksh, T.L., 1993. Spinal nitric oxide synthesis inhibition blocks NMDA-induced thermal hyperalgesia and produces antinociception in the formalin test in rats. Pain 54, 291–300.

Malmberg, A.B., Yaksh, T.L., 1995. The effect of morphine on formalin-evoked behaviour and spinal release of excitatory amino acids and prostaglandin E_2 using microdialysis in conscious rats. Br. J. Pharmacol. 114, 1069–1075.

Marotta, P., Sautebin, L., Di Rosa, M., 1992. Modulation of the induction of nitric oxide synthase by eicosanoids in the murine macrophage cell line J774. Br. J. Pharmacol. 107, 640–641.

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N.E., Schatz, A.R., Gopher, A., Almog, S., Martin, B.R., Compton, D.R., et al., 1995. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem. Pharmacol. 50, 83–90.

Milano, S., Arcoleo, F., Dieli, M., D'Agostino, R., D'Agostino, P., De Nucci, G., Cillari, E., 1995. Prostaglandin E_2 regulates inducible nitric oxide synthase in the murine macrophage cell line J774. Prostaglandins 49, 105–115.

Minami, T., Uda, R., Horiguchi, S., Ito, S., Hyodo, M., Hayaishi, O., 1994. Allodynia evoked by intrathecal administration of prostaglandin E_2 to conscious mice. Pain 57, 217–223.

Minami, T., Nakano, H., Kobayashi, T., Sugimoto, Y., Ushikubi, F., Ichikawa, A., Narumiya, S., Ito, S., 2001. Characterization of EP receptor subtypes responsible for prostaglandin E_2-induced pain responses by use of EP1 and EP3 receptor knockout mice. Br. J. Pharmacol. 133, 438–444.

Misko, T.P., Schilling, R.J., Salvemini, D., Moore, W.M., Currie, M.G., 1993. A fluorometric assay for the measurement of nitrite in biological samples. Anal. Biochem. 214, 11–16.

Pang, L., Hoult, J.R., 1997. Repression of inducible nitric oxide synthase and cyclooxygenase-2 by prostaglandin E_2 and other cyclic AMP stimulants in J774 macrophages. Biochem. Pharmacol. 53, 493–500.

Pertwee, R.G., 2001. Cannabinoid receptors and pain. Prog. Neurobiol. 63, 569–611.

Pestonjamasp, V.K., Burstein, S.H., 1998. Anandamide synthesis is induced by arachidonate mobilizing agonists in cells of the immune system. Biochim. Biophys. Acta 1394, 249–260.

Rehse, K., Ciborski, T., 1995. New no-donors with antithrombotic and vasodilating activities, X: Antiplatelet and antithrombotic effects of 3-methylsydnone-5-nitrosimine (RE 2047) in combination with ASA, pentoxifylline, and ticlopidine. Arch. Pharm. (Weinheim) 328, 71–78.

Richardson, J.D., Aanonsen, L., Hargreaves, K.M., 1998. Antihyperalgesic effects of spinal cannabinoids. Eur. J. Pharmacol. 345, 145–153.

Salvemini, D., Settle, S.L., Masferrer, J.L., Seibert, K., Currie, M.G., Needleman, P., 1995. Regulation of prostaglandin production by nitric oxide; an in vivo analysis. Br. J. Pharmacol. 114, 1171–1178.

Shibuta, S., Mashimo, T., Zhang, P., Ohara, A., Yoshiya, I., 1996. A new nitric oxide donor, NOC-18, exhibits a nociceptive effect in the rat formalin model. J. Neurol. Sci. 141, 1–5.

Smith, F.L., Fujimori, K., Lowe, J., Welch, S.P., 1998. Characterization of delta9-tetrahydrocannabinol and anandamide antinociception in nonarthritic and arthritic rats. Pharmacol. Biochem. Behav. 60, 183–191.

Sugiura, T., Kobayashi, Y., Oka, S., Waku, K., 2002. Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance. Prostaglandins Leukot. Essent. Fatty Acids 66, 173–192.

Swierkosz, T.A., Mitchell, J.A., Warner, T.D., Botting, R.M., Vane, J.R., 1995. Co-induction of nitric oxide synthase and cyclo-oxygenase: interactions between nitric oxide and prostanoids. Br. J. Pharmacol. 114, 1335–1342.

Szczeklik, A., Gryglewski, R.J., Czerniawska-Mysik, G., 1975. Relationship of inhibition of prostaglandin biosynthesis by analgesics to asthma attacks in aspirin-sensitive patients. Br. Med. J. 1, 67–69.

Taiwo, Y.O., Levine, J.D., 1990. Effects of cyclooxygenase products of arachidonic acid metabolism on cutaneous nociceptive threshold in the rat. Brain Res. 537, 372–374.

Tjolsen, A., Berge, O.G., Hunskaar, S., Rosland, J.H., Hole, K., 1992. The formalin test: an evaluation of the method. Pain 51, 5–17.

Ueno, A., Matsumoto, H., Naraba, H., Ikeda, Y., Ushikubi, F., Matsuoka, T., Narumiya, S., Sugimoto, Y., Ichikawa, A., Oh-ishi, S., 2001. Major roles of prostanoid receptors IP and EP(3) in endotoxin-induced enhancement of pain perception. Biochem. Pharmacol. 62, 157–160.

Vetter, G., Geisslinger, G., Tegeder, I., 2001. Release of glutamate, nitric oxide and prostaglandin E_2 and metabolic activity in the spinal cord of rats following peripheral nociceptive stimulation. Pain 92, 213–218.

Walker, J.M., Huang, S.M., Strangman, N.M., Tsou, K., Sanudo-Pena, M.C., 1999. Pain modulation by release of the endogenous cannabinoid anandamide. Proc. Natl. Acad. Sci. U. S. A. 96, 12198–12203.

Weinberg, J.B., 2000. Nitric oxide synthase 2 and cyclooxygenase 2 interactions in inflammation. Immunol. Res. 22, 319–341.

Welch, S.P., Dunlow, L.D., Patrick, G.S., Razdan, R.K., 1995. Characterization of anandamide-and fluoroanandamide-induced antinociception and cross-tolerance to delta 9-THC after intrathecal administration to mice: blockade of delta 9-THC-induced antinociception. J. Pharmacol. Exp. Ther. 273, 1235–1244.

White, R., Ho, W.S., Bottrill, F.E., Ford, W.R., Hiley, C.R., 2001. Mechanisms of anandamide-induced vasorelaxation in rat isolated coronary arteries. Br. J. Pharmacol. 134, 921–929.

Yaksh, T.L., Malmberg, A.B., 1994. Central pharmacology of nociceptive transmission. In: Wall, P.D., Melzack, R. (Eds.), Textbook of Pain. Churchill Livingstone, Edinburgh, pp. 165–200.

Zimmer, A., Zimmer, A.M., Hohmann, A.G., Herkenham, M., Bonner, T.I., 1999. Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. Proc. Natl. Acad. Sci. U. S. A. 96, 5780–5785.

Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.

Zygmunt, P.M., Chuang, H., Movahed, P., Julius, D., Hogestatt, E.D., 2000. The anandamide transport inhibitor AM404 activates vanilloid receptors. Eur. J. Pharmacol. 396, 39–42.