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HU-210 shows higher efficacy and potency than morphine after intrathecal administration in the mouse formalin test

Hans Gühring [*,1], Johannes Schuster [1], May Hamza, Mehmet Ates, Carolin Edith Kotalla, Kay Brune

Department of Experimental and Clinical Pharmacology and Toxicology, Fahrstrasse 17, D-91054 Erlangen, Germany

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Abstract

The discovery of endocannabinoids opens up new perspectives in experimental pain research. Here we present data for the excellent antinociceptive properties of the synthetic cannabinoid, *R*(−)-7-hydroxy-delta-6-tetra-hydrocannabinol-dimethylheptyl (HU-210), after intrathecal and oral administration in mice. It is known that cannabinoids depress motor activity. Therefore, these compounds are suspected of influencing antinociceptive tests. Our behavioural tests (RotaRod, tail flick) clearly show that HU-210 affects nociceptive behaviour even at dosages which do not yet influence motor activity. Moreover, spinal microdialysis (5 μl/min) in the dorsal horn of freely moving mice showed an enhancement of prostaglandin production during the formalin test. HU-210 applied via artificial cerebral spinal fluid during microdialysis perfusion increases prostaglandin concentrations under both baseline and formalin test conditions. Indomethacin reduces the HU-210 effect on pronociceptive prostaglandin production but does not reinforce the antinociceptive properties of HU-210. Thus, HU-210 shows antinociceptive properties that are independent of its influence on the prostaglandin pathway. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Despite recent advances in the understanding of the neurobiology of pain, the pharmacologic targets and tools for pain relief remain the same: there are antipyretic anti-inflammatory analgesics, which block the generation of pronociceptive prostaglandins from the arachidonic acid cascade or antipyretic analgesics, which do not show anti-inflammatory properties, and opiates acting on the endogenous opioid system. No effective alternatives to opiates have so far been available to relieve severe afferent pain.

To relieve pain, cannabis has been used for thousands of years. The discovery of the endocannabinoid system resulted in a renaissance of scientific interest in cannabinoid-induced antinociception. It is now known that the mammalian tissues contain at least two types of $G_{i/0}$ coupled cannabinoid receptors, CB_1 (cloned in 1990) and CB_2 (cloned in 1993). Cannabinoid CB_1 receptors are expressed mainly in neurones of the central and peripheral nervous system, whereas CB_2 receptors occur centrally and peripherally in certain non-neuronal tissues (for review, see Pertwee, 1997; 1998). In laboratory animals, the analgesic effect of different tetrahydrocannabinol analogues is well documented (Pertwee, 2001). When tetrahydrocannabinol analogues were administered peripherally, they depressed motor activity in doses similar to that at which they are effective in pain tests (Kosersky et al., 1973; Martin et al., 1995). Therefore, these compounds are suspected of influencing antinociceptive tests. In this context, it is important that the cannabinoid CB_1 receptor was found, among other localizations in the spinal cord (Sanudo-Pena et al., 1999). Intrathecally injected cannabinoid receptor agonists were able to reduce pain-related behaviour in different pain models and in different laboratory animals (a summary was given by Pertwee, 2001). There is a line of evidence that the antinociceptive effects of cannabinoids are at least in part CB_1-receptor-mediated (Adams and Martin, 1996; Ledent et al., 1999; Lichtman et al., 1996; Welch et al., 1995; Zimmer et al., 1999).

* Corresponding author. Tel.: +49-9131-85-26937; fax: +49-9131-85-22-77-4.

E-mail address: guehring@pharmakologie.uni-erlangen.de (H. Gühring).

[1] Contributed equally in this work.

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Prostaglandins are thought to act as pronociceptive tissue mediators. It is known that subcutaneous injection of formalin (Geisslinger et al., 2000; Muth-Selbach et al., 1999) or zymosan (Guhring et al., 2001; Tegeder et al., 2001) into one hind paw resulted in higher prostaglandin levels in the spinal cord. The prostaglandin-producing enzymes are targets of the antipyretic anti-inflammatory analgesics (e.g. indomethacin).

In this context, it is surprising that tetrahydrocannabinol stimulates arachidonic acid mobilization (Hunter and Burstein, 1997; Reichman et al., 1988) via activation of phospholipase A_2 (Hunter et al., 1986; Wartmann et al., 1995). Increased levels of prostaglandins are the consequence of the arachidonic acid mobilization (Bhattacharya, 1986; Fimiani et al., 1999; Hunter et al., 1991).

Consequently, the present experiments were performed to compare the antinociceptive effects of the synthetic cannabinoid, HU-210, to those of morphine as the golden standard. The influence of HU-210 on spinal prostaglandin production was evaluated both under basal conditions and during the formalin test.

2. Materials and methods

2.1. Experimental animals

For all experiments (expect for microdialysis for which C57 black mice were used), male mice of the B6/129/SF2 strain weighing between 18 and 22 g were used (the original breeder pairs were obtained from Jackson Laboratories, Bar Harbor, ME, USA, stock: #101045).

Animals were housed in groups and were allowed free access to food and water until the start of the experiment. All behavioural experiments were conducted during the period between 10:00 and 13:00 h with normal room light and temperature (21 ± 1 °C).

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

2.2. Drug administration

R(−)-7-hydroxy-delta-6-tetra-hydrocannabinol-dimethylheptyl (HU-210; L04524) as a cannabinoid receptor agonist, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methy l-1*H*-pyrazole-3-carboxamide (AM-251; L06261) as an analogue of the cannabinoid receptor antagonist, SR 141716A, which binds with high affinity to the cannabinoid CB_1 receptor (Gatley et al., 1996; Gatley et al., 1997) and *R*-1-Methanandamide (L15312d) as a cannabinoid receptor agonist were obtained from Alexis (Grünberg, Germany). Indomethacin (L67H1609) as a non-selective cyclooxygenase inhibitor was obtained from Sigma (Deisenhofen, Germany). For comparison, morphine hydrochloride was obtained from Merck (Darmstadt, Germany). All drugs were dissolved and stored in ethanol, Tween 80 (P1754, Sigma). All suspensions for the intrathecal injections were prepared daily and contained 1% black ink (to control for correct injection site after termination of the experiments), 10% ethanol, 1% Tween 80 and artificial cerebral spinal fluid (ACSF; consisting of 151.1 mM Na^+, 2.6 mM K^+, 0.9 mM Mg^{2+}, 1.3 mM Ca^{2+}, 122.7 mM Cl^-, 21.0 mM HCO_3^-, 2.5 mM HPO_4^- and 3.5 mM dextrose, pH 7.2; ACSF was bubbled with carbogen (5% CO_2/95% O_2)). For the subcutaneous injections (50 μl), no black ink was used and phosphate-buffered saline replaced ACSF. For the microdialysis study, the ACSF contained 0.05% ethanol and 0.1% Tween 80.

2.3. Application of drugs

Intrathecal (i.t.) injections were performed essentially as described elsewhere (Hylden and Wilcox, 1980). In brief, mice were anaesthetized with isoflurane (1.5–2.0 vol.%, Abbott, Wiesbaden, Germany) and 1 μl of drug containing solutions or vehicle (ACSF) was injected into the spinal subarachnoid space between L5 and L6 35 min prior to the administration of formalin using a 26-gauge needle mated to a 5-μl Hamilton syringe. Mice showing neurological abnormalities were excluded (fewer than 3%). One percent black ink (Pelikan) was added to all solutions used for i.t. injections. Proper siting of i.t. injections was verified by inspection of slices of the spinal cord after lumbar laminectomy.

2.4. Experimental procedure

Drugs were administered after tail flick and RotaRod testing. After 30 min, the animal were examined for neurological abnormalities and retested in the tail flick and RotaRod apparatus. Approximately 35 min after the drug administration, formalin was injected subcutaneously. The tail flick and RotaRod examination were repeated following the formalin test.

2.5. Formalin-induced nociception test

Nociceptive behaviour was assessed using the formalin test essentially as described by Dubuisson and Dennis (1977). The behavioural parameter suggestive of nociception was paw flinching (rapid shaking of the injected paw) as it is regarded to be a consistent and robust component of formalin-induced behaviour. Briefly, formalin (5%, 20 μl) was injected subcutaneously into the dorsal surface of the left hind paw. The numbers of paw flinchings were recorded for a period of 60 min. Prior to the injection of formalin, the animals were placed in the acrylic observation cage for approximately 30 min in order to allow acclimatization.

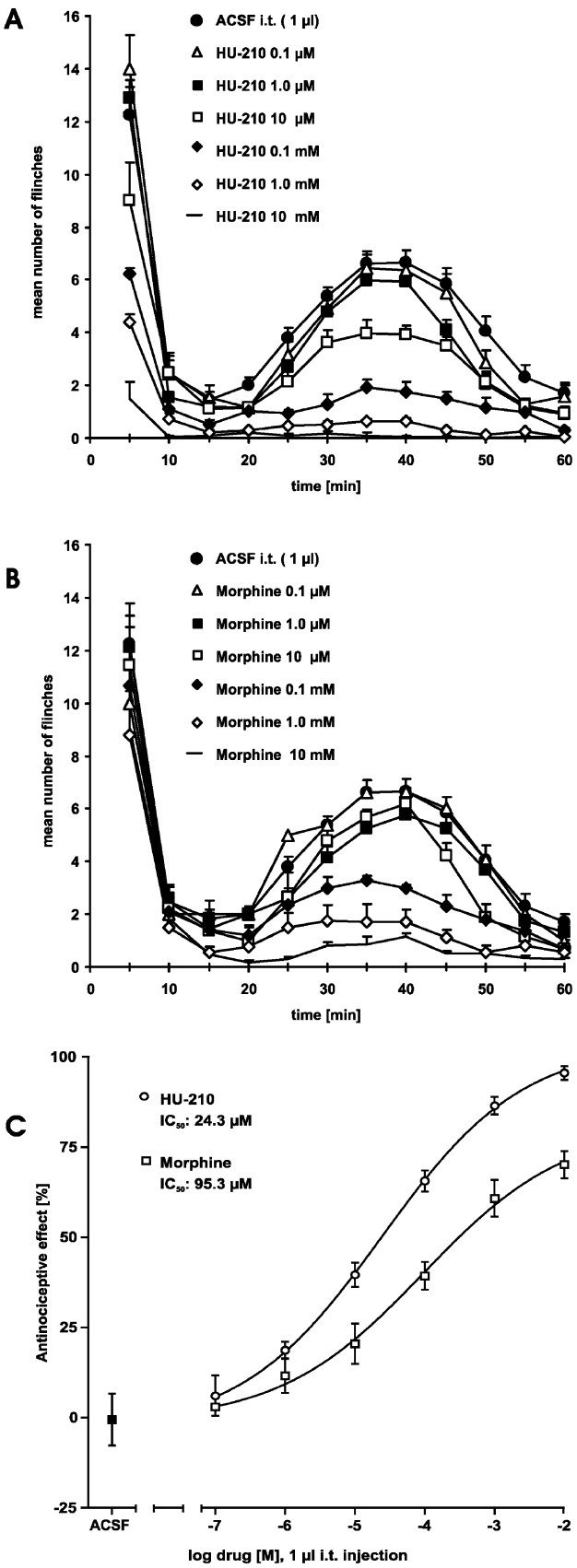

Fig. 1. Comparison of the antinociceptive effects of the synthetic cannabinoid HU-210 and of morphine in the mouse formalin test after intrathecal drug administration (only 1 µl was injected intrathecally into the thoracolumbal bone segment). A shows the dose-dependent reduction of the spontaneous flinching behaviour by HU-210 30 min after intrathecal drug application. B shows the antinociceptive effects of morphine under the same conditions. C summarizes the results of A and B by a curve-fit of the antinociceptive properties of both drugs. Data are expressed as means ± S.E.M.

2.6. RotaRod and tail flick test

The RotaRod technique was introduced by Dunham and Miya in 1957. It has proven to be of great value in research to test drugs for their potential effects on motor coordination or fatigue resistance. The day before the formalin test, mice were trained on the RotaRod apparatus (model 7650; Ugo Basile, Italy). For mice, training consisted of 5 to 10 subsequent 1-min attempts on a rod rotating at 12 rpm. Mice were tested again on the morning of the experiment. Only animals that managed to stay on the rod for 1 min at least once were used in the experimental procedure. After baseline assessment, drugs were given. Drop latencies were re-measured 30 min after drug administration and after completion of the formalin test.

The tail flick test, which is a thermal analgesia measurement method for rodents (D'Amour and Smith, 1941) is considered to quantify a spinal reflex reaction. Tail flick latencies are measured by a tail flick test apparatus (model 7360, IR intensity 15, Ugo Basile). The mean of the tail flick latencies measured in three pre-drug trials was taken as the individual baseline. Only animals showing tail flick latencies ranging from 7 to 10 s before treatments were used in the experiments. Drugs were given after baseline assessment. Tail flick latencies were re-measured 30 min after drug administration and after completion of the formalin test.

2.7. Spinal microdialysis

The dialysis tube was constructed from a Cuprophan hollow fiber (outer diameter 216 µm) with a 36-kDa molecular weight cut-off (Hospal, Nürnberg, Germany). This fiber was connected on one side to a polyethylene (PE) tube (inner diameter 0.4 mm, outer diameter 0.8 mm) using cyanacrylate glue (No 448 Stabiloplast Renfert Chemietechnik). A small metal spike was inserted into the other side and fixed with quick-setting cyanacrylate glue (UHU, Bühl, Germany).

Mice were deeply anaesthetized with isoflurane (1.5–2.0 vol.%, Abbott, Wiesbaden, Germany) and placed on an electronically controlled heating pad (37 °C, CMA/Microdialysis, Stockholm, Sweden). After cutaneous incision of the thoracolumbar region, superficial and deep dorsal lumbar fascia were slit and muscle tissue was removed from

the vertebrae Th_{12}–L_1. The dialysis tube was introduced through the intervertebral joints between the thoracic and lumbar bones. All accessible parts of the dialysis tube were covered with cyanacrylate glue. After cutting the spike, the free end of the hollow fiber was connected to another PE tube using the same cyanacrylate adhesive. Afterwards the mice were sewn up and placed in the observation chamber. Mice showing neurological abnormalities were excluded (fewer than 5%). Animals were allowed free access to food and water and usually started eating within 15 min after waking up from anaesthesia. An additional large PE tube was used as a holder for the small PE tubes from the microdialysis to protect the tubes from being scratched off.

The PE tube was connected to a microdialysis pump (CMA 100, CMA/Microdialysis) and ACSF was perfused at a flow rate of 5 μl/min. Samples were collected at 15-min intervals in Eppendorf cups kept on ice and finally stored at −70 °C for subsequent analysis of prostaglandin E_2. After a washout period of 180 min, the treatment started with either vehicle or drug administration over 30 min. Then three baseline samples were collected at 15-min intervals. Following baseline collections, 20 μl of formalin (5%) was injected s.c. into the left hind paw as in the behaviour experiments and five samples were collected. Following killing of the mice, proper placement of microdialysis tubes was verified by perfusion with black ink (Pelikan) and subsequent microscopic examination.

2.8. Determination of prostaglandin E_2

Aliquots (15 μl) of the microdialysis perfusion 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, cali-

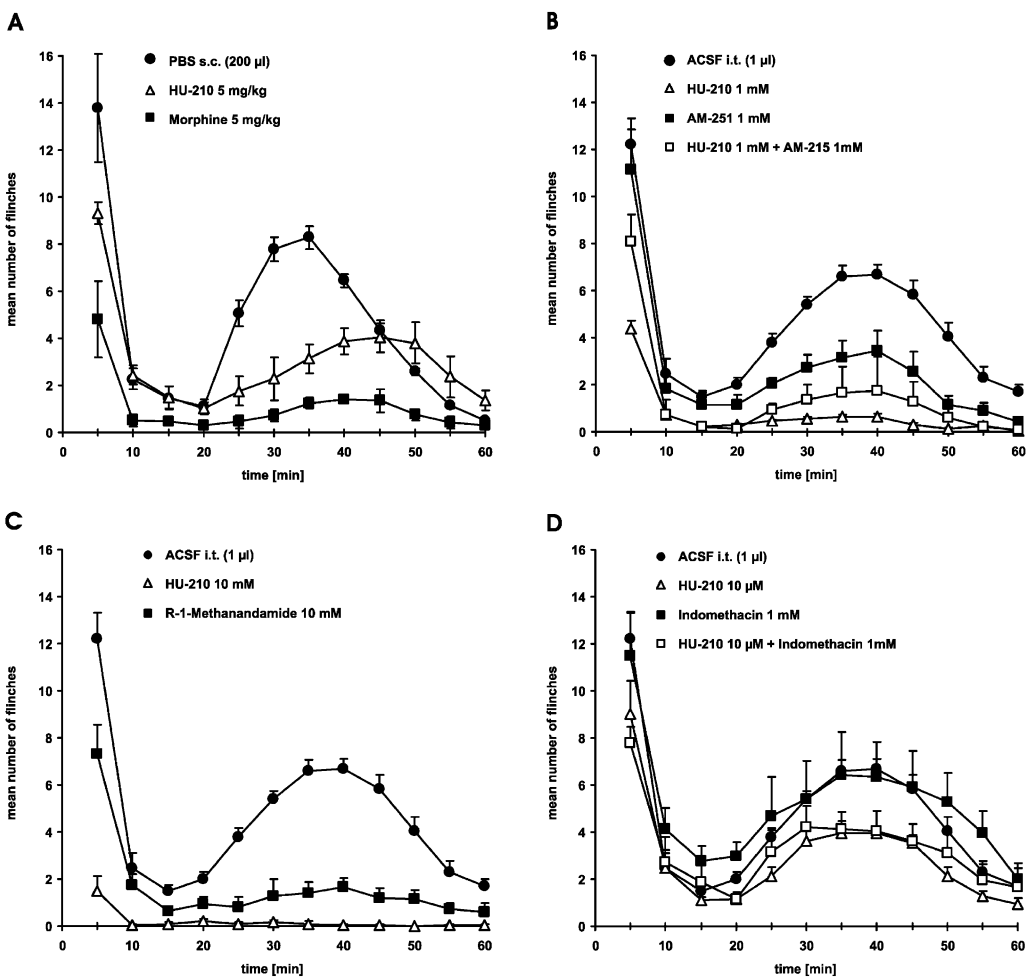

Fig. 2. A shows the effects of HU-210 and morphine after subcutaneous injection of 5 mg/kg in comparison to those of the drug vehicle (phosphate-buffered saline with 0.05% Tween 80 and 0.1% ethanol). B shows that at least part of the antinociceptive effect of HU-210 is mediated by the cannabinoid CB_1 receptor, as the CB_1-receptor antagonist, AM-251, is able to abolish the antinocicepive effect of HU-210 and itself acts as a partial antagonist. C compares the effects of HU-210 and of *R*-1-methanandamide after intrathecal injection. D shows that indomethacin has no antinociceptive effect after intrathecal injection of 1 μl of a 1-mM solution. No additional effects are seen in combination with HU-210. Data are expressed as means ± S.E.M.

bration range: 1000–7.8 pg/ml (Cayman Chemicals, Ann Arbor, MI, USA). Measurements were completed using an enzyme-linked immunosorbent assay reader (DIAS Microplate Reader, Dynatech Laboratories, Great Britain) with an absorbency maximum at 405 nm.

2.9. Data analysis

The IC_{50} value (the concentration producing 50% of the maximal inhibitory effect) for the antinociceptive action was obtained by fitting the data points representing the total number of flinches after formalin injection over 60 min for 5–8 animals per group by a non-linear least-squares method using the Graph Pad Prism® software version 3.0. The maximal effect is equal to suppression of all flinches. Statistical analyses were carried out using one-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni test (the α level was set to 0.05) or Student's *t*-test ($P < 0.05$ was considered statistically significant). All results are expressed as means ± S.E.M.

3. Results

3.1. Formalin test

As expected, mice showed the two characteristic phases of nociceptive behaviour in the formalin test; approximately 15 min after formalin application an initial high flinch period (first or early phase) followed by a lull, was followed by a more prolonged but delayed second (or late) phase. After intrathecal injection of 1 μl of the drug solution, HU-210 or morphine, a significant difference in the total numbers of flinches as compared to the vehicle group up to 10 or 100 μM was found, respectively (Fig. 1A–B). Overall, HU-210 showed a higher efficacy and potency than morphine, with IC_{50} values from 24.3 μM for HU-210 and 95.3 μM for morphine (Fig. 1C). Interestingly, HU-210 was much more efficient than morphine in the first phase of the formalin test. Both drugs were able to reduce the spontaneous pain-related flinching behaviour in

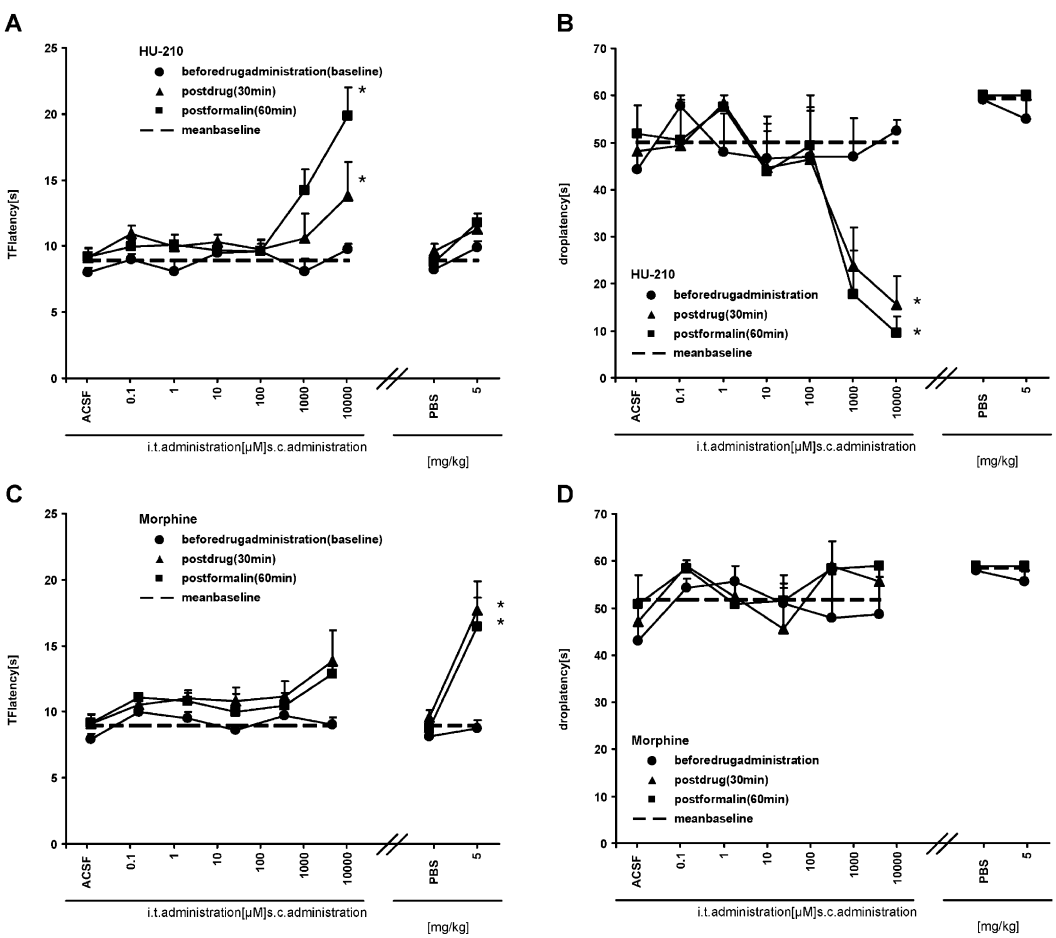

Fig. 3. A shows that the intrathecal injection of HU-210 altered the tail flick latencies after injection of 1 mM drug solution, but did not change the latencies after subcutaneous drug application of 5 mg/kg. C shows that morphine did not alter the tail flick latencies until 10 mM drug application but increased them more pronouncedly after subcutaneous drug application as compared to HU-210. B and C show the RotaRod performance after application of HU-210 and morphine as a marker for motor impairment. Whereas morphine did not provoke any motor alterations, HU-210 significantly increased motor insufficiency after intrathecal injection of 1 mM. Data are expressed as means ± S.E.M. * $P < 0.05$ compared with vehicle control.

a dose-dependent manner in both phases of the test. After subcutaneous administration of HU-210 and morphine (5 mg/kg) both drugs showed antinociceptive properties (Fig. 2A). Surprisingly, AM-251 as a cannabinoid CB_1-receptor antagonist showed antinociceptive properties after intrathecal injection but reduced the effect of HU-210 when the compound was injected in combination with HU-210. In our experiments, AM-251 acts as a partial antagonist (Fig. 2B). The next experiment was performed to verify the cannabinoid action of another compound in the spinal cord. The use of the endogenous cannabinoid analogue, *R*-1-Methanandamide, showed the same antinociception as did HU-210 after intrathecal injection (Fig. 2C). As previously shown, the cyclooxygenase inhibitor, indomethacin, is an effective drug in the formalin test even after intrathecal injection (Chapman and Dickenson, 1992; Malmberg and Yaksh, 1992). In our experiments, the injection of 1 μl of a 1-mM solution failed to alter the flinching behaviour. The combination of HU-210 and indomethacin did not change the effect of HU-210 (Fig. 2D), indicating that the antinociceptive properties of HU-210 are not linked with the prostaglandin pathway.

3.2. Behavioural experiments

Cannabinoids are known to depress motor activity. To clear antinociceptive effects of HU-210 of this suspicion we measured spinal cord reflex activity in the tail flick test and examined the animals' coordination on the RotaRod. As shown in Fig. 3A, up to 100 μM of HU-210, no difference was observed in the tail flick latency. Significant differences were found after intrathecal injection of 1 μl of a 10-mM suspension. RotaRod performance was influenced by HU-210 after 1 mM (Fig. 3B). No drug effect on the tail flick latency or RotaRod performance

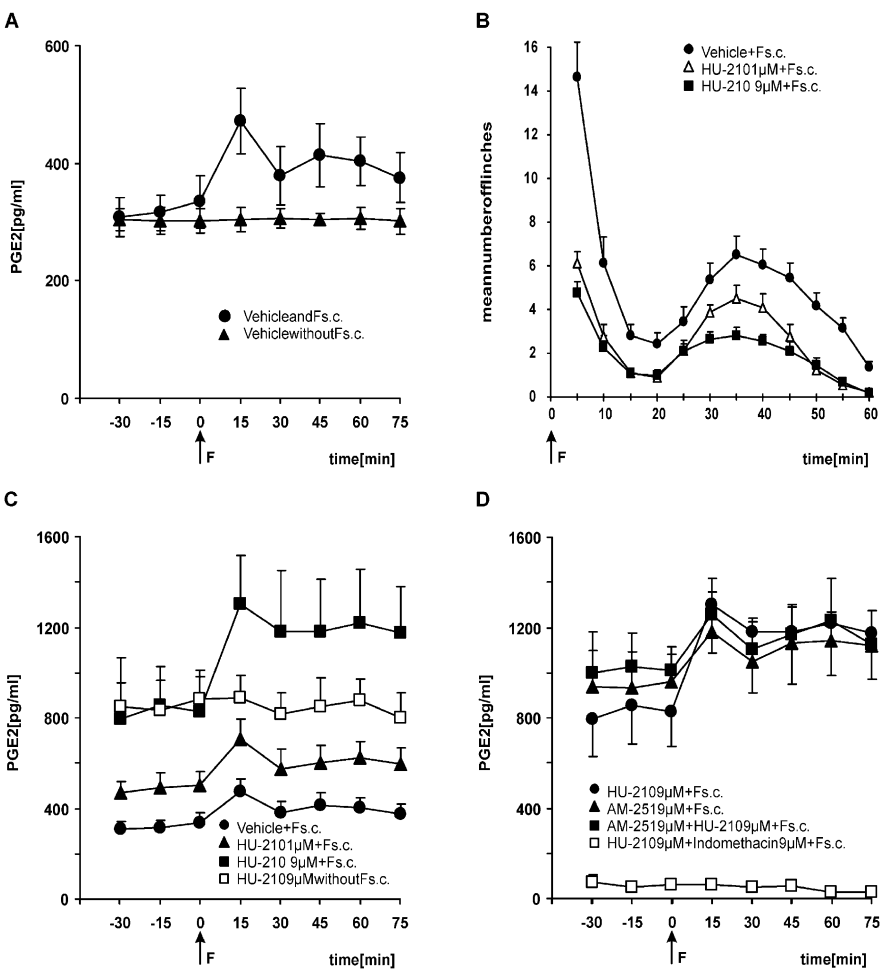

Fig. 4. A–D show the results of the microdialysis study. A shows that subcutaneous injection of formalin resulted in elevated prostaglandin release in the lumbar 4–5 segment of the spinal cord. B shows an antinociceptive effect of HU-210 in the freely moving mice in the formalin test during microdialysis 3.5 h after completion of the operating procedure. C shows that HU-210 is able to increase both baseline and formalin-evoked release of prostaglandin E_2 in the spinal cord. D shows that the enhancement of prostaglandin E_2 by HU-210 is not CB_1-receptor-mediated, as the CB_1-receptor antagonist was not able to reduce prostaglandin E_2 release. Additionally, indomethacin as a control reduced prostaglandin E_2 production in combination with HU-210. "F" indicates the time of subcutaneous formalin injection. Data are expressed as means ± S.E.M.

was observed after peripheral application of HU-210 (5 mg/kg). In contrast to HU-210, morphine did not influence behaviour at the dosages used in either behavioural test; only after peripheral administration (5 mg/kg) was a significant increase in tail flick latencies recorded (Fig. 3C–D).

3.3. Microdialysis experiments

These experiments were performed with freely moving mice. This means that the animals can move in the observation cage and have free access to water and food. After surgery the animals showed no signs of abnormalities. Only the typical postoperative scratching behaviour was present. Operated B6/129/SF2-mice showed the typical flinching pattern with the two phases of the nociceptive response after formalin application (Fig. 4B) as did non-operated animals (Fig. 1A). In the two drug groups (1 and 9 μM), HU-210 was applied via the microdialysis probe. Formalin was injected after a 60-min perfusion period with the ACSF-vehicle or ACSF-drug. These experiments again showed that HU-210 acts dose dependently as a antinociceptive drug in the spinal cord (Fig. 4B). The outflow of the microdialysis probe was collected and analysed for the amount of prostaglandin E_2 which is considered an important pronociceptive mediator within the spinal cord. As expected, formalin injection led to a sharp increase of the prostaglandin E_2 amount (approximately 50% increase) within the first 15 min (Fig. 4A). We found that the administration of HU-210 resulted in a dose-dependent increase of prostaglandin E_2 production. Interestingly, higher prostaglandin levels were already found in the baseline values (30 min after treatment) and were stable over the entire observation period. In general, perfusion with 9 μM HU-210 significantly increased prostaglandins. It is important that HU-210 did not change the percent increase of the prostaglandin values after formalin injection (45% to 50%, Fig. 4C). The following experiments were designed as control experiments. The mixture of HU-210 9 μM and indomethacin 9 μM reduced, as expected, the prostaglandin values. Surprisingly, like HU-210, the cannabinoid CB_1 receptor antagonist, AM-251, itself enhanced the prostaglandin production. Additionally, AM-251 failed to reduce the HU-210-driven prostaglandin production (Fig. 4D).

4. Discussion

The course of the behavioural response to formalin in mice is well known: it shows an early or first phase immediately after injection as well as a late or second phase starting approximately 15–20 min after injection and lasting for 20–40 min. The two phases differ qualitatively in that the first phase seems to be caused mainly by C-fibre activation due to the peripheral stimulus, whereas the second phase is believed to involve inflammatory processes and functional changes in the spinal cord (for review, see Tjolsen et al., 1992).

We now compared the antinociceptive properties of the synthetic cannabinoid, HU-210, to those of morphine after intrathecal administration. There was a dose-dependent antinociceptive effect produced by the cannabinoid, HU-210. This compound reaches a higher efficacy than does morphine. A critical point in this observation is that at dosages from 1 to 10 mM, the compound diminishes motor ability in the RotaRod test and increases the tail flick latencies. However, the fact that the dose–response curve does not show a kink eliminates this criticism. The dose-dependent effect of HU-210 in the formalin test argues strongly for a receptor-mediated process. It is known that there are cannabinoid CB_1 receptors in the spinal cord (Farquhar-Smith et al., 2000) and that HU-210 in the spinal cord acts as an agonist on the CB_1 receptor (Drew et al., 2000). AM-251 was designed as an analogue to the highly selective CB_1 receptor antagonist, SR 141716A. In our experiments, this compound showed only partial antagonistic effects in the formalin test and no antagonism to activation of the arachidonic acid cascade by HU-210. It is so far unclear whether this activation of the arachidonic acid cascade is a receptor-mediated process or not.

In the microdialysis experiments, HU-210 enhanced prostaglandin E_2 production in a dose-dependent manner. It is well known that prostaglandins are not stored in the cells but are produced by the cyclooxygenases. Therefore, it is clear that the administration of HU-210 resulted in rapid activation of the arachidonic acid cascade. As a control, indomethacin was applied in combination with HU-210 and strongly reduced prostaglandin production as expected. In order to estimate the contribution of the prostaglandins to the nociceptive response in the formalin test, we injected indomethacin only and in combination with HU-210. Intrathecal injection of indomethacin failed to reduce the nociceptive response provoked by formalin at the chosen dosages (1 μl of a 1-mM solution = 1 nmol). Previously, it was shown that intrathecal administration of indomethacin to rats reduced the flinching behaviour with an ID_{50} of 1.9 nmol (Malmberg and Yaksh, 1992). In these latter experiments, rats were chronically intrathecally cannulated. It may be possible that the cannulation changes the response to formalin (Almeida et al., 2000). However, in our experiments with mice, the combination of indomethacin and HU-210 did not increase the antinociceptive properties of HU-210.

In conclusion, the findings reported here suggest that the synthetic HU-210 has a genuine analgesic action in the formalin model of tonic nociceptive pain. This analgesia appears to be mediated by cannabinoid CB_1 receptors and is not influenced by the production of pronociceptive prostaglandins.

Acknowledgements

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