The CGRP receptor antagonist BIBN4096BS peripherally alleviates inflammatory pain in rats

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A B S T R A C T

Calcitonin gene–related peptide (CGRP) is known to play a major role in the pathogenesis of pain syndromes, in particular migraine pain. Here we focus on its implication in a rat pain model of inflammation, induced by injection of complete Freund adjuvant (CFA). The nonpeptide CGRP receptor antagonist BIBN4096BS reduces migraine pain and trigeminal neuronal activity. Here we demonstrate that the compound reduces inflammatory pain and spinal neuronal activity. Behavioural experiments reveal a reversal of the CFA-induced mechanical hypersensitivity and monoiodoacetate (MIA)-induced weight-bearing deficit in rats after systemic drug administration. To further investigate the mechanism of action of the CGRP antagonist in inflammatory pain, in vivo electrophysiological studies were performed in CFA-injected rats. Recordings from wide dynamic range neurons in deep dorsal horn layers of the lumbar spinal cord confirmed a reduction of neuronal activity after systemic drug application. The same amount of reduction occurred after topical administration onto the paw, with resulting systemic plasma concentrations in the low nanomolar range. However, spinal administration of BIBN4096BS did not modify the neuronal activity in the CFA model. Peripheral blockade of CGRP receptors by BIBN4096BS significantly alleviates inflammatory pain.

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1. Introduction

The neuropeptide calcitonin gene–related peptide (CGRP) is well known to play an important role in the pathophysiology of pain. In particular, the relevance of CGRP in migraine pain has been extensively studied preclinically [3,26,70] and clinically [8,13,34,65]. CGRP receptor antagonists such as BIBN4096BS were developed to selectively bind human CGRP receptors [61]. BIBN4096BS abolishes increases in dural blood flow [66] and facial skin blood flow evoked by trigeminal stimulation in marmosets [11] and rats [35]. It was the first CGRP receptor antagonist to be tested in clinical trials [59], and it proved to be efficacious in migraine treatment [10]. The emerging novel therapeutic class of CGRP antagonists is effective in treating migraine attacks [18,51,57].

CGRP receptors are expressed in several brain regions [33,40,62,67] and the spinal cord [7,73]. In dorsal horn neurons of the spinal cord, they are colocalized and functionally connected with AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors [21], indicating their involvement in transmission and modulation of sensory information from primary afferents. CGRP was demonstrated to participate in central sensitization of pain pathways. It appears to be an important signaling molecule along the peptidergic nociceptive pathway from primary afferents and the spinal cord to supraspinal regions [1]. In the amygdala of normal rats, CGRP administrations facilitate synaptic transmission [23]. Systemically or spinally applied, CGRP-receptor antagonists reduce neuronal activity in the amygdala [1], in the trigeminal nucleus [19], in and the spinal cord [29] in rat pain models.

Little is known about functional effects of CGRP antagonism on spinal neurotransmission with blockade of solely peripheral CGRP receptors. A systemic distribution does not exclude peripheral drug activity, which cannot be separated from central activity. We aimed at a local but not spreading drug distribution. A CGRP receptor blockade restricted to peripheral sites can be achieved by topical drug application on the skin with penetration of the drug through the skin but neglectably low systemic distribution in the plasma. Topical and systemic administration of ketanserin, a 5-HT2A receptor antagonist demonstrated to decrease CGRP levels in dorsal root ganglia, attenuates hypersensitivity in neuropathy [69]. These data allude to an involvement of peripheral CGRP receptors in neuropathic pain.

In addition, the proinflammatory neuropeptide CGRP has also been implicated in the generation and maintenance of peripheral inflammation [36,39]. In this study, we assessed the involvement of CGRP receptors in inflammatory pain (complete Freund...
adjuvant, CFA). In addition, the influence of CGRP receptor inhibition on pain has been tested in a model for osteoarthritic pain (monosodium acetate, MIA). The site of analgesic action has been studied by measuring spinal neuronal activity in the inflammatory pain model induced after intravenous, spinal, and topical application of BIBN4096BS. Effects of BIBN4096BS were compared to those of ketorolac, a nonsteroidal anti-inflammatory drug (NSAID) used as positive control.

2. Methods

2.1. Animals

Male Wistar rats (300–400 g body weight, Charles River Laboratories, USA) were housed in group cages under a 12 h light/dark cycle with food and water ad libitum in institutional animal facilities. All housing conditions and experimental procedures were approved by the German federal government and supervised by the institution’s department of animal welfare. Experiments followed the guidelines of the German Animal Welfare Act 2006 and adhered to the guidelines of the Committee for Research and Ethical Issues of IASP 1983. All efforts were made to minimize animal suffering and to reduce the number of animals used.

CFA was injected subcutaneously in 1 hind paw (50 L) to induce an acute inflammation. Experiments were done 24 h after CFA injection. Surgeries were done under isoflurane anaesthesia and followed procedures described earlier [30,58]. The control group of animals was naive to therapy and received no injections into the paw.

2.2. Behavioural testing

2.2.1. CFA pain model

Twenty-four hours after CFA injection into the paw and 1 h after systemic drug or vehicle application, mechanical sensitivity was assessed by a Randall-Selitto test using an electromechanical algesimeter (Ugo Basile, Italy) measuring withdrawal thresholds upon increasing pressure applied to the hind paw. The weight force (g) exerted on the paw, provoking the first sign of pain, was noted as individual pain threshold, and the animal was released immediately. Drugs were given intraperitoneally (i.p.) or subcutaneously (s.c.). Groups were statistically compared to the vehicle group.

2.2.2. Osteoarthritis model

Rats were anaesthetized with a 2% isoflurane–oxygen mixture and received a single intra-articular injection in the right knee of 50 L of 1 mg MIA (Calbiochem, Germany) dissolved in physiological saline. The left contralateral control knee was injected with 50 L of saline.

On day 2 after MIA injection, rats were trained for the measurement of weight-bearing deficit (ie, difference in hind paws weight distribution between the MIA- and saline-injected paws) with a modified incapacitation test apparatus (Boehringer Ingelheim, Germany).

At measurement, the force exerted by each hind paw was averaged over a 10 s period. To assess the weight bearing on the next day (day 3 after MIA), a baseline measurement of deficit was performed (0 h time point). Rats were allocated in groups such that baseline weight-bearing deficits were equally distributed in the testing groups.

Rats then received BIBN4096BS at the dose of 3 mg/kg (s.c.), morphine at 6 mg/kg s.c., or vehicle alone. Weight-bearing deficit was assessed at 1, 2, and 4 h after compound administration.

2.3. Spinal cord electrophysiology

Electrophysiological studies were conducted 24 h after CFA injection. Animals were anesthetized with pentobarbital, with 90 mg/kg i.p. initially, then kept on a 30 mg/kg/h infusion rate intravenously (i.v.) during the whole experiment. One of each arterial carotis, vena jugularis, and vena femoralis were cannulated for blood pressure control, anaesthesia, and drug application, respectively. A tracheal tube was used for artificial respiration (UNO Micro Ventilator; UNO Roestvastaal BV, Netherlands) if needed. The body temperature of the rat was maintained at 37°C rectally using a heated plate and infrared light. By laminectomy, the L4–6 segmental region of the spinal cord receiving afferent input from the hind paw was exposed. Extracellular recordings of deep wide dynamic range (WDR) neurons (300–600 mV) with defined receptive fields in the hind paw were performed with a tungsten electrode (6 MΩ; FHC, USA), a differential amplifier (DAM80; WPI, USA), and a data-capturing interface with software (Notocord-hem 3.5; Notocord Systems, France). All neurons responding to light touch and noxious stimuli (pinch, noxious heat), to cold (4°C), and to dynamic (brush) stimuli. The spontaneous activity (frequency of action potentials) was quantified over a period of 40 s before stimulation.

Electronic von Frey stimulations (EvF; filament diameter 1.0 mm; Somedic, Sweden) were applied to the plantar surface of the hind paw, and resulting neuronal activity from WDR neurons was recorded. The stimulation interval was 10 min. Each stimulation consisted of 3 mechanical stimuli at 3 stimulation strengths: innocuous (10 g EvF, 3 × 5 s each), intermediate (30 g EvF, 3 × 5 s), and noxious (100 g EvF, 3 × 5 s) stimuli. Frequencies of action potentials were measured on plateau phases of stimuli. Baseline conditions of at least 3 stable control responses were achieved for each parameter before drug application.

2.4. Determination of plasma levels

Total plasma levels of BIBN4096BS were determined by liquid chromatography/mass spectrometry using a Triple Quadrupole system (API 4000; Sciei, Canada). Samples were taken from arterial blood (heparin) at 5, 15, 25, and 35 min after drug application onto the paw (topical) or after i.v. application.

2.5. Drugs

For behavioural studies, drugs were dissolved in 0.9% NaCl and applied i.p. or s.c. 1 h before Randall-Selitto testing (BIBN4096BS, 1, 3, and 10 mg/kg s.c.; ketorolac trometamol, 10 mg/kg i.p.). For electrophysiological experiments, polyethylene glycol 200 (PEG) was added. Drugs were applied i.v. (1% PEG), spinally (5% PEG) onto the exposed spinal cord, and topically (30% PEG) on the receptive field of the paw onto the skin. Drug effects on neuronal activity were followed 60 min until recovery. The activity during the first 3 stimuli within 30 min after drug application was pooled because peak effects occurred at 5–25 min after drug application.

Drug concentrations and volumes were adapted from literature if data were available for the different application routes, as follows: BIBN4096BS, 60 mM (3 mg/60 L) topically, 1 mM (1 µg/µL) spinally [19], 1 mg/kg i.v. [19,38]; and ketorolac trometamol, 200 mM (3 mg/60 L) topically, 6 mM (100 µg) spinally, 10 mg/kg i.v. Application of 3 mg ketorolac in topical formulations was demonstrated to be effective in rats [17]. Vehicle was administered as 30% PEG/NaCl topically, 5% PEG/NaCl spinally, 1% PEG/NaCl i.v.
2.6. Statistical analysis

For statistical analysis, Prism5 (GraphPad Software, USA) was used, with its MannWhitney test and Student’s t test. Significance levels were taken to be \( P < .05 \) and \( P < .01 \).

3. Results

3.1. The CGRP antagonist BIBN4096BS reduces inflammatory pain

Mechanical hypersensitivity of the paw 24 h after CFA-induced inflammation was measured by the Randall-Selitto paw pressure test (Fig. 1A). One hour after subcutaneous vehicle application, withdrawal thresholds were 219 ± 8 g. This mechanical hypersensitivity was alleviated 1 h after 10 mg/kg intraperitoneal ketorolac application (336 ± 21 g). Subcutaneous BIBN4096BS application with doses >1 mg/kg also significantly reduced mechanical hypersensitivity: 10 mg/kg BIBN4096BS s.c. completely reversed it (380 ± 12 g) to the thresholds seen in naive animals (400 ± 10 g).

3.2. BIBN4096BS was detectable in the plasma after topical administration

Plasma concentrations of BIBN4096BS were determined in CFA animals after i.v. and topical drug administration. They drop from 800 nM to 100 nM within 35 min after 1 mg/kg i.v. BIBN4096BS application (Fig. 1B, upper curve). After topical application of 60 mM BIBN4096BS on the skin of the paw, plasma levels were 4–1 nM at 5–25 min after application (Fig. 1B, lower curve), suggesting penetration through the skin.

3.3. BIBN4096BS reduces osteoarthritic pain

Mechanical hypersensitivity was tested in a model of osteoarthritis where MIA had been injected into knee (Fig. 1C). After baseline measurements, rats received BIBN4096BS at the dose of 3 mg/kg (s.c.), morphine at 6 mg/kg (s.c.), or vehicle. Weight-bearing deficit was assessed at 1, 2, and 4 h after compound administration. BIBN4096BS demonstrated a long-lasting reversal of weight-bearing deficit (\( P < .01 \)) at 1 and 2 h after dosing, its effect was comparable to the positive control, morphine. At 4 h, although not statistically significant from vehicle, BIBN4096BS still demonstrated a partial reversal of weight-bearing deficit in MIA rats (26 ± 8 g vs. 43 ± 12 g for BIBN4096BS and vehicle-treated group, respectively; \( P = .1 \)). The morphine-treated group maintained a significant improvement of weight-bearing deficit up to 4 h after dosing (21 ± 4 g for morphine-treated group; \( P < .05 \) vs vehicle-treated group).

3.4. Electrophysiological effects of BIBN4096BS on WDR neurons

Spinal neuronal activity of WDR neurons was recorded extracellularly, evoked by mechanical stimulation of the receptive field in the CFA-inflamed or untreated paw. Action potential frequencies before drug application were normalized to 100% as a baseline response. Drug effects were compared to baseline responses after i.v., topical, and spinal applications (Fig. 1C). Neuronal activity was reduced by BIBN4096BS and ketorolac in CFA animals, not in naive controls. Prominent drug effects of BIBN4096BS were detected after topical drug application onto the skin of the paw, giving a 35% reduction of the basal level. Similarly, intravenously applied BIBN4096BS reduces neuronal activity by 33%. Spinally applied onto the exposed spinal cord, BIBN4096BS lacked effectiveness in our setting. In contrast, ketorolac reduced neuronal activity after spinal administration by 16% of basal response, similar to a 17% reduction after i.v. administration. The NSAID, however, lacked efficacy after topical application.

To study the drug effects of BIBN4096BS and ketorolac in CFA-induced inflammation in detail, 4 neuronal response parameters were examined: spontaneous activity; and mechanically evoked activity during a light innocuous stimulus (10 g EvF), an intermediate stimulus (30 g EvF), and a strong noxious stimulus (100 g EvF). Drugs were applied intravenously, topically, or spinally onto the exposed spinal cord.

3.5. Intravenous application of BIBN4096BS and ketorolac reduce spinal neuronal activity in CFA

Intravenously applied, 1 mg/kg BIBN4096BS reduces evoked neuronal activity in the CFA pain model (Fig. 2A). The frequency of spontaneous activity of WDR neurons is transiently reduced after stimulation but recovers within few minutes. Mechanically
same at all stimulus strengths tested. Ketorolac was ineffective approximately 60% of control response during low, medium, and high mechanical stimulations. BIBN4096BS, however, reduced the neuronal activity to approximately 60% of control levels few minutes later. Spontaneous activity, measured 40 s before stimulation, was unaltered after vehicle, BIBN4096BS, or ketorolac application in CFA animals (Fig. 3B). Vehicle and ketorolac did not change spinal firing frequencies during mechanical stimulation of all strengths after topical drug administration. BIBN4096BS did not reduce the ipsilaterally recorded firing frequency after contralateral drug application on the noninflamed paw (Fig. 3C), suggesting a local peripheral action of the drug. In naive animals, vehicle and BIBN4096BS had no effect (Fig. 3D).

3.7. Spinal administration of BIBN4096BS on the exposed spinal cord is without electrophysiological effect in CFA

Local administration of 1 mM BIBN4096BS onto the exposed spinal cord did not significantly change spinal activity (Fig. 4A). Spontaneous firing and evoked activity were unaltered after BIBN4096BS application in CFA (Fig. 4B) and naive animals (Fig. 4C), while 6 mM ketorolac significantly reduced evoked activity in CFA rats (Fig. 4B).

4. Discussion

4.1. The CGRP antagonist BIBN4096BS reduces inflammatory pain

The involvement of CGRP in migraine pain is well documented [9, 10]. Its impact on inflammatory pain, however, has not yet been studied intensively. Here we demonstrate that the nonpeptide CGRP receptor antagonist BIBN4096BS reverses mechanical hyperalgesia induced by CFA injection into the paw in rats. Drugs were applied systemically in the behavioral experiment, resulting in high plasma concentrations of BIBN4096BS. After topical drug application on the skin, plasma levels of BIBN4096BS were detectable (1–4 nM), suggesting a transdermal delivery of the drug. Given a Ki of 3 nM and low brain penetration of the compound [11, 25], it is unlikely that effective levels in the central nervous system could be reached. Rather, a locally restricted effect of the drug is assumed. BIBN4096BS concentrations known to produce central nervous system effects are in the micromolar [1] or millimolar ranges [19].

4.2. Local topical application of BIBN4096BS reduces WDR neuronal activity

Additionally to behavioral experiments and plasma level determinations, the effects of BIBN4096BS on WDR neuronal activity were studied after systemic, topical, and spinal administration to define the predominant site of action for this CGRP receptor antagonist in the CFA inflammatory pain model. Spinal neuronal activity is reduced by BIBN4096BS, indicating an analgesic effect at early stages of the pain pathway by interfering with signal transduction at the first synapses. As is known from the trigeminal system, central and peripheral CGRP receptors control neuronal activity in normal rats [49, 66]. However, CGRP receptor localization suggests multiple targets for CGRP in the trigeminovascular system [33], including involvement in presynaptic regulation of nociceptive transmission, as well as indirect modulation of peripheral neuronal activity via suppression of inflammatory cell activity.

The present data support the hypothesis that blockade of peripheral CGRP receptors yields efficient pain control in a model reflecting inflammatory pain. Our electrophysiological experiments reveal a reduction of evoked spinal neuronal activity by BIBN4096BS after systemic or topical drug application. Although resulting plasma levels differ greatly between the 2 application routes, the electrophysiological effect was the same. Intravenously applied, BIBN4096BS reduced WDR neuron activity at both high and low stimuli, suggesting an inhibitory effect towards a wide range of nociceptive mechanical stimuli. Behaviorally, efficacy was observed against high mechanical stimulations of all strengths after topical drug administration. BIBN4096BS did not reduce the ipsilaterally recorded firing frequency after contralateral drug application on the noninflamed paw (Fig. 3C), suggesting a local peripheral action of the drug. In naive animals, vehicle and BIBN4096BS had no effect (Fig. 3D).
threshold (as measured by the Randall–Selitto test in the CFA model) and less intense threshold (as measured by weight bearing deficit in the MIA model), confirming the broader analgesic profile of BIBN4096BS in rat pain models. The compound was equally effective after topical administration indicating the periphery is the critical site of action for efficacy of the CGRP antagonist in the inflammatory pain condition.

Topically applied, BIBN4096BS exerts an inhibitory effect on WDR neurons, most likely blocking only peripheral CGRP receptors. This is confirmed by the low levels of the compound in the plasma circulation—values below the concentration needed to block the rat CGRP1 receptor [11]. There is evidence that CGRP is present and is mediating nociceptive stimuli in the skin [31,44,56] and the sole of the foot (glabrous skin) of rats [12]. Intraplantar injection of CGRP can induce hypersensitivity in mice [50], and CGRP antagonists can block capsaicin-induced hypersensitivity when applied to the skin [48]. Our electrophysiological and pharmacokinetic data suggest that the skin, and therefore peripheral nerve endings, are the main site of action for BIBN4096BS to target CGRP receptors. It remains to be determined which cell type is the target for the CGRP receptor blocker in inflammatory pain models.

CGRP is well known for its role on vasodilatation in the skin [28] via activation of its receptors on endothelial cells. A close contact between CGRP fibers and blood vessels was found in inflamed subcutaneous tissue [52]. Huang et al. [32] demonstrated that CGRP can also modulate the release of nociceptive-related chemokines from endothelial cells. Accordingly, this effect would lead to a decrease in inflammatory and pain mediators and therefore cannot explain the analgesic properties of CGRP receptor antagonist observed in our studies. Immune cells and neurons [2,43,74] express CGRP receptors. CGRP can be released by macrophages, and CGRP receptor antagonist can decrease the release of proinflammatory mediators from immune cells [47]. Ex vivo electrophysiology studies demonstrated that CGRP facilitated synaptic transmission and that the selective CGRP1 receptor antagonist CGRP8–37 can reverse synaptic plasticity in spinal neurons from arthritic rats [5]. However, there is no consolidated evidence demonstrating a direct effect of CGRP to stimulate peripheral nerves.

Overall, this evidence supports the hypothesis that the CGRP system can regulate peripheral nociception in inflamed tissues acting at the level of nerve terminals via direct inhibition of proinflammatory mediators from inflammatory cells, and that inhibition of vasodilatation induced by BIBN4096BS can contribute to reduce the infiltration of inflammatory cells to the site of injury. Nevertheless, we cannot fully exclude the notion that the antinociceptive properties of BIBN4096BS could derive from a direct inhibition of CGRP function at neuronal levels. More studies should be performed to further understand the neurobiology of CGRP receptors at the levels of peripheral nerve fiber endings.

Because the effect on WDR neuronal activity after topical application of BIBN4096BS is as strong as after systemic administration, our data suggest a predominantly peripheral site of action, most likely at the skin level. Spontaneous neuronal discharge was reduced only transiently after stimulation, whereas a sustained effect of the compound on basal neuronal activity was reported for trigeminal neurons [19]. The reduction of firing seen here was short lasting. Therefore, it was considered to not effectively alter spontaneous activity.

In naive animals, the drug has no effect on WDR neurons, congruent with the findings on central neurons [1]. The NSAID ketorolac exerted spinal electrophysiological effects in the CFA pain model. These data agree with NSAIDs being spinal active [20,22].

We compared BIBN4096BS effects to ketorolac because NSAIDs are the preferred therapeutic option to treat inflammatory pain, and ketorolac is one of the most widely used NSAIDs in the clinical setting [71]. However, its gastrointestinal side effects reduce its usefulness. CGRP antagonists proved to be much safer [10], providing good analgesia without NSAID-typical dyspepsia, gastric ulceration, or bleeding. Ketorolac has been demonstrated to be largely ineffective against pain when provided spinally to humans [14–16]. However, in animal pain models, intrathecal ketorolac produces analgesia [72]. This suggests a more limited role for spinal cord cyclooxygenase in human pain states than predicted.
by studies in animals. It remains unknown whether this mismatch, which reveals a certain pain-related mechanism between humans and rodents, can also be attributed to the CGRP system. The opioid system, for example, is believed to be very similar and thus predictive [46,60]. Close consistency between clinical and preclinical pharmacological results has also been demonstrated after spinal administrations [27,42,53]. Our data from the CFA and the MIA pain models affirm the hypothesis of BIBN4096BS targeting peripheral CGRP receptors, confirming that for early inflammatory conditions, the impact of peripheral CGRP receptors exceeds the impact of spinal receptors [45,64]. In our model, BIBN4096BS is more potent in reducing spinal neuronal activity after topical application than the NSAID ketorolac. Similarly, higher doses of ketorolac were required in behaviour to reverse hyperalgesia. However, there might be limitations to the interpretation of ketorolac's effects [37,41,68]. Although it has been demonstrated to be effective in inflammation [17], we cannot exclude the notion that in our setting, it did not penetrate the skin sufficiently. In particular, the CFA pain model was reported to require up to 10× higher NSAID doses [6] or chronic treatment [4] to demonstrate ketorolac sensitivity. This might be attributed to model singularities. Surprisingly, BIBN4096BS failed to reduce WDR neuronal activity after spinal application. It was demonstrated to have a central effect when applied locally to the spinal cord in an arthritis model [1]. The kaolin- or carrageenan-induced inflammation of the knee joint, however, was studied at its plateau [24,54,55]. The development of the inflammatory process and the time course of its centralization might be different in the CFA model, whereas CGRP levels were found to decline within the first 2 days after CFA injection in the dorsal horn before they rise above control levels during the next 8 days [63]. At our time point of investigation, 24 h after CFA injection, peripheral CGRP effects might thus have exceeded central effects.

Spinal CGRP receptors also appear to be involved in the generation and maintenance of capsaicin-induced inflammatory pain [64]. Compared to this model, central effects of CGRP antagonism again seem to require several more days when inflammation is induced by intraplantar injection of CFA [45]. In contrast to our CFA model, the capsaicin model is known to rapidly induce central sensitization [36]. Thus, the lack of BIBN4096BS being spinal active in CFA might be a specificity of this model.

In summary, our behavioural results demonstrate the potential of pain reduction by the CGRP antagonist BIBN4096BS in inflammatory pain models. The in vivo electrophysiological experiments suggest a predominantly peripheral site of action. This indicates an important participation of peripheral CGRP receptors in the CFA inflammatory rat pain model. These receptors can successfully be targeted by BIBN4096BS delivered transdermally. Unlike systemic drug administration, this local administration circumvents adverse effects.

Conflict of statement

The authors report no conflict of interest.

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