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Enhancement of the effects of a complete inhibitor of enkephalin-catabolizing enzymes, RB 101, by a cholecystokinin-B receptor antagonist in diabetic rats

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1 RB 101, a complete inhibitor of enkephalin-catabolizing enzymes, has been previously shown to produce antinociception in normal rats after systemic administration. Moreover, its coadministration with a cholecystokinin-B (CCK-B) receptor antagonist has been shown to strongly enhance its antinociceptive effect in normal rats. In this work, we determined whether RB 101 was able to reduce hyperalgesia and allodynia in diabetic rats, a model of neuropathic pain. The type of opioid receptors (μ or δ) involved was determined using naloxone and naltrindole, respectively, and the interactions between endogenous enkephalins and CCK on nociception control was investigated using coadministration of RB 101 and the CCK-B receptor antagonist CI-988.

2 RB 101 suppressed mechanical hyperalgesia (paw pressure-induced vocalization test), partially alleviated mechanical allodynia (von Frey hair test), and was ineffective in thermal allodynia (tail immersion test). The analgesic effect was completely cancelled by naloxone or naltrindole, suggesting that is requires the availability of μ- and/or δ-opioid receptors.

3 The combination of an inactive dose of CI-988 with the lowest effective dose of RB 101 resulted in a stronger increase in the vocalization threshold comparatively to RB 101 alone.

4 The present study demonstrates that the antinociception generated by RB 101 induced by elevation of extracellular levels of endogenous enkephalins, can be extended to neuropathic pain in diabetic rats and that blockade of CCK-B receptors potentiated antinociceptive effects elicited by RB 101.

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Abbreviations: CCK, cholecystokinin

Introduction

The analysis of the possible pathophysiological differences in neuropathic pain and particularly the influence of the etiology of the disorders on the involvement of any system modulating the nociceptive message motivated this study. There is much evidence that hyperalgesic states resulting from neuropathic pain are associated with modified efficacy of opiate drugs. Thus, pharmacological studies have indicated that, on a molar basis, morphine is half as potent in streptozocin-induced diabetic rats as in healthy rats (Courteix *et al*., 1994). Basic works have suggested a surexpression of anti-opioid systems, particularly CCK-ergic system to explain this lower efficacy (Faris *et al*., 1983; Noble *et al*., 1993). Furthermore, it has been suggested that protection of endogenous opioids from degradation may provide analgesia with reduced side effects comparatively to exogenous opiates (review in Roques *et al*., 1993). Accordingly, we studied the effects of systemic administered RB 101, a compound able to block the enkephalin-catabolizing enzymes and to cross the blood–brain–barrier (Fournié-Zaluski *et al*., 1992; Noble *et al*., 1992), in streptozocin-induced diabetes, a model of neuropathic pain (Courteix *et al*., 1993). Indeed, this inhibitor induces potent antinociceptive responses in normal and mononeuropathic rats after systemic administration (Noble *et al*., 1992; Xu *et al*., 1997) by elevating the extracellular levels of enkephalins (Ruiz-Gayo *et al*., 1992; Daugé *et al*., 1996). As RB 101 has never been studied in diabetes-induced neuropathic pain, the present work was carried out to study the effects of this dual inhibitor on mechanical hyperalgesia and allodynia, and on thermal allodynia in diabetic rats. Furthermore, the involvement of opioid receptors has been assessed using naloxone and naltrindole, μ- and δ-opioid receptor antagonists, respectively.

In addition, it could be of interest to study the effects of the combination of a CCK-B receptor antagonist: CI-988 with RB 101. Actually, it has been reported that antinociception induced by systemic RB 101 is strongly potentiated by selective CCK-B receptor antagonists on carrageenin-induced

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c-Fos expression, in the rat tail flick test and the mouse hot-plate, on the flexor reflex in decerebrate, spinalized rats (Maldonado *et al*., 1993; Valverde *et al*., 1994; Honoré *et al*., 1997; Xu *et al*., 1997), indicating a functional antagonism between the endogenous opioidergic and CCK-ergic systems in modulating nociception (Noble *et al*., 1992). Therefore, the effects of CI-988, a CCK-B receptor antagonist (Hughes *et al*., 1990), on the antinociceptive responses induced by RB 101 were determined using the paw pressure test in diabetic rats.

Methods

Animals

Male Sprague-Dawley rats (Charles River, France) were housed four per cage under standard laboratory conditions, and given food and water *ad libitum*. As some suffering might result from these experiments, the I.A.S.P. Committee for Research and Ethical Guidelines (Zimmermann, 1983) were followed. Great care was taken, particularly with regard to housing conditions, to avoid or minimize discomfort of the animals. The animals were kept on solid floored cages with a deep layer of sawdust to accommodate the excess of urination and cages were changed daily.

Induction of diabetes

Rats (200–250 g) were intraperitoneally injected with streptozocin (75 mg kg^{-1}) (Zanosar®, Upjohn, France) dissolved in distilled water. Diabetes was confirmed 1 week later by measurement of tail vein blood glucose levels with Ames Dextrostix® and a reflectance colorimeter (Ames Division, Miles Laboratories, France). Only rats with a final blood glucose level of at least 14 mM were included in the study. This animal model of chronic pain with mechanical, thermal and chemical hyperalgesia has been described in detail by Courteix *et al*. (1993).

Pain behaviour testing

Assessment of mechanical hyperalgesia The rats were submitted to the paw pressure test previously described by Randall & Selitto (1957). Nociceptive thresholds, expressed in grams, were measured using a Ugo Basil analgesimeter (Apelex, tip diameter of probe 1 mm, weight 30 g) by applying increasing pressures to the left hind paw until vocalization was elicited (maximal pressure was set up at 750 g).

Assessment of mechanical allodynia Rats were individually placed on an elevated plastic mesh (1 cm^2 perforations) in a clear plastic cage and were adapted to the testing environment for at least 15 min. Von Frey hairs (VFH) (Semmes-Weinstein monofilaments, Stoelting, IL, U.S.A.) with calibrated bending forces (1.479, 2.041, 3.630, 5.495, 8.511, 11.749, 15.136 and 28.840 g) were used to deliver punctuate mechanical stimuli of varying intensity. The VFH were applied to the plantar surface of each hindpaw, from below the mesh floor. Each stimulus was applied for a duration of approximately 1 s with an interstimulus interval of approximately 5 s. Care was taken to stimulate random locations on the plantar surface. Paw withdrawal threshold was defined as the minimum pressure required to elicit a withdrawal reflex of the paw, at least on time on the five trials. Then, with the next filaments of increasing diameter, the same withdrawal reflex (between 1/5 and 5/5) was expected to consider the response positive. Voluntary movement associated with locomotion was not taken as a withdrawal response. Only robust and immediate withdrawal responses produced by the stimulus were considered. Mechanical allodynia was defined as a significant decrease in withdrawal thresholds to VFH application. The 28.840 g hair was selected as the upper limit cut-off for testing.

Assessment of thermal allodynia The tail of the rats was immersed in a water bath maintained at 42°C, a temperature which is normally innocuous in normal rats, until tail withdrawal or signs of struggle were observed (cut-off time 15 s) (Courteix *et al*., 1993). A shortened duration of immersion indicates allodynia.

Drugs

RB 101: {N-[R,S)-2-benzyl-3[(S)(2-amino-4-methylthio)butyl dithio]-1-oxo-propyl]-L-phenylalanine benzyl ester} (mesylate salt), an inhibitor of enkephalin degrading-enzymes was synthesized as described (Fournié-Zaluski *et al*., 1992). It was dissolved in ethanol (10%), cremophor EL (10%), and distilled water (80%) on the day of the experiment. Cremophor was purchased from Sigma Chemical (Saint-Quentin-Fallavier, France).

CI-988: 4-{[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.$1^{3,7}$] dec-2-yloxy) carbonyl]amino] propyl] amino]-1-phenylethyl] amino}-4-oxo[R-(R*,R*)]-butanoate, N-methyl-D-glucamine, a CCK-B receptor antagonist, was a generous gift from Prof Hughes (Parke-Davis, Cambridge, U.K.). It was dissolved in saline (0.9% NaCl) on the day of the experiment.

Naloxone and naltrindole were purchased from RBI (Bioblock, Illkirsch, France) and dissolved in saline on the day of the experiment.

Injections

RB 101 and its vehicle, naloxone, and saline were administered intravenously in a caudal vein, in a volume of 1 ml kg^{-1}.

CI-988, naltrindole and saline were injected intrathecally in the subarachnoid space between L5 and L6 in a volume of 10 μl using a 30½ Ga needle and a 25 μl Hamilton syringe as described by Mestre *et al*. (1994).

Experimental design

Tests took place 4 weeks after the induction of diabetes. At this time, only rats in which the reduction of pain scores was more than 15% of the value obtained before the streptozocin injection were included (i.e., about 75% of the diabetic rats). These animals were submitted to the paw pressure test before drug injection. Once two stable threshold values were obtained, drugs were injected. Allodynic rats (i.e., diabetic

rats in which showed an aversive reaction to the application of a non painful mechanical or thermal stimulus) were also submitted to the test before and after the drug injection.

Drugs were injected as follows:

Experiment 1: RB 101 (5, 10, 20, and 40 mg kg^{-1}, i.v.) or vehicle (1 ml kg^{-1}, i.v.) to test their effects on mechanical hyperalgesia and mechanical and thermal allodynia.

Experiment 2: Naloxone (0.5 mg kg^{-1}, i.v.) or naltrindole (1 μg rat^{-1}, i.t.) 15 min before RB 101 (20 mg kg^{-1}, i.v.) to test their effects on mechanical hyperalgesia.

Experiment 3: CI-988 (0.1 μg rat^{-1}, i.t.) 5 min before the lowest effective dose of RB 101 (5 mg kg^{-1}, i.v.) to appreciate the effect of the combination on mechanical hyperalgesia.

The vocalization thresholds were measured in mechanical hyperalgesia each 15 min for 120 min following the injections. For mechanical and thermal allodynia, the withdrawal thresholds and tail immersion durations were measured each 15 min for 60 min following the injections. All the experiments included for each treatment were performed blind ($n=8$) using a randomized block to avoid any chronobiological effects, and to assess the effects of the different treatments under the same environmental conditions.

Statistical analysis

The paw withdrawal and vocalization thresholds in grams were expressed as means $\pm$ s.e.mean of raw data. The mean areas under the vocalization threshold $-$ time curves were calculated with the trapezoidal rule, using the program Siphar/Win (1-2b, SIMED, Créteil, France). Statistical significance was assessed using two-way analysis of variance (ANOVA) followed, when the *F*-value was significant, by a Dunnett's test to determine the time course of the effect of various treatments on the paw withdrawal threshold or by Student's *t*-test for unpaired series to compare the AUCs. To analyse the time course of the mechanical allodynia, differences between groups were assessed using the Mann–Whitney *U*-test. The significance level was set up at $P<0.05$.

Results

The body weight of some of the rats decreased after the induction of diabetes by streptozocin and therefore the weakest animals exhibiting a loss of weight of more than 10% were systematically removed from the study and sacrificed. The rats used in all the treatment groups exhibited a mean weight of 300 ± 20 g at the time of use. Four weeks after the induction of diabetes by streptozocin treatment, the vocalization thresholds were significantly reduced in 75% of the animals (317 ± 11 g before induction *versus* 162 ± 10 g after induction), which corresponds to a mechanical hyperalgesia. In allodynic experiments, pre-diabetes thresholds raised the cut-off after the application of innocuous mechanical (28.840 g) or thermal (15 s) stimulus. Four weeks after streptozocin injection, the withdrawal threshold to VFH application (2.90 ± 0.39 g) and the reaction time to tail withdrawal (8.83 ± 1.00 s) significantly decreased in all the diabetic animals.

Effects of RB 101 or vehicle alone (Experiment 1)

Mechanical hyperalgesia RB 101 produced a significant dose-dependent antinociceptive effect resulting in a complete suppression of diabetes-induced hyperalgesia and an antinociceptive effect for the doses of 20 and 40 mg kg^{-1} ($P<0.001$) (Figure 1a). The maximal antinociceptive effect corresponded to a vocalization threshold elevation of $+294\pm28$ g (i.e., $+178\pm9\%$ of the predrug score) for the dose of 40 mg kg^{-1} at 20 min, not significantly different from the maximal effect obtained after 20 mg kg^{-1}. The effect of RB 101 was characterized by a short duration for the lowest dose of 5 mg kg^{-1} (20 min) and lasted 60 min for the other three doses of 10, 20 and 40 mg kg^{-1}. The corresponding AUCs confirmed the dose-dependent antinociceptive effect of RB 101, and the ceiling effect at the dose of 40 mg kg^{-1} (Figure 1b).

Mechanical and thermal allodynia RB 101 induced a significant increase in withdrawal threshold only for the dose of 40 mg kg^{-1} (maximal increase: $+8.3\pm4.0$ g at 20 min) ($P<0.01$). This effect lasted 20 min (Figure 1c).

None of the four doses significantly affected reaction time to tail immersion (data not shown).

Effects of naloxone and naltrindole on the antinociceptive effect of RB 101 on mechanical hyperalgesia (Experiment 2)

The μ-opioid receptor antagonist naloxone injected at the dose of 0.5 mg kg^{-1} i.v. 15 min before the injection of RB 101 (20 mg kg^{-1}, i.v.) entirely suppressed the antinociceptive effect produced by RB 101 at the dose of 20 mg kg^{-1} (Figure 2a).

Similarly, the i.t. injection of a δ-opioid receptor antagonist naltrindole at the dose of 1 μg rat^{-1}, suppressed the antinociceptive effect of RB 101 (20 mg kg^{-1}, i.v.) (Figure 2b).

Effect of the combined administration of CI-988 and RB 101 on mechanical hyperalgesia (Experiment 3)

Since the effect of i.t. CI-988 and i.v. RB 101 (5 mg kg^{-1}) occurred at 15 (Coudoré-Civiale *et al*., 2000) and 10 min, respectively, the injection of CI-988 was given 5 min before that of RB 101. The combination of CI-988 0.1 μg rat^{-1} i.t. with RB 101 5 mg kg^{-1} i.v. significantly increased the vocalization thresholds from the 10th min to the 30th min following the injection (Figure 3). This combination exerted an antinociceptive effect with a maximal score elevation of $+263\pm21$ g (i.e., $+137\pm8\%$ of the predrug score) at 10 min ($P<0.001$), significantly higher than those produced by CI-988 alone or by RB 101 alone (maximal score elevation of $+69\pm5$ g, i.e., $+42\pm5\%$ of the predrug score).

Discussion

In the present study, a mixed inhibitor of enkephalin degrading enzymes, RB 101, was shown, when systemically administered to diabetic rats, to be able to suppress the mechanical hyperalgesia produced by paw pressure and to

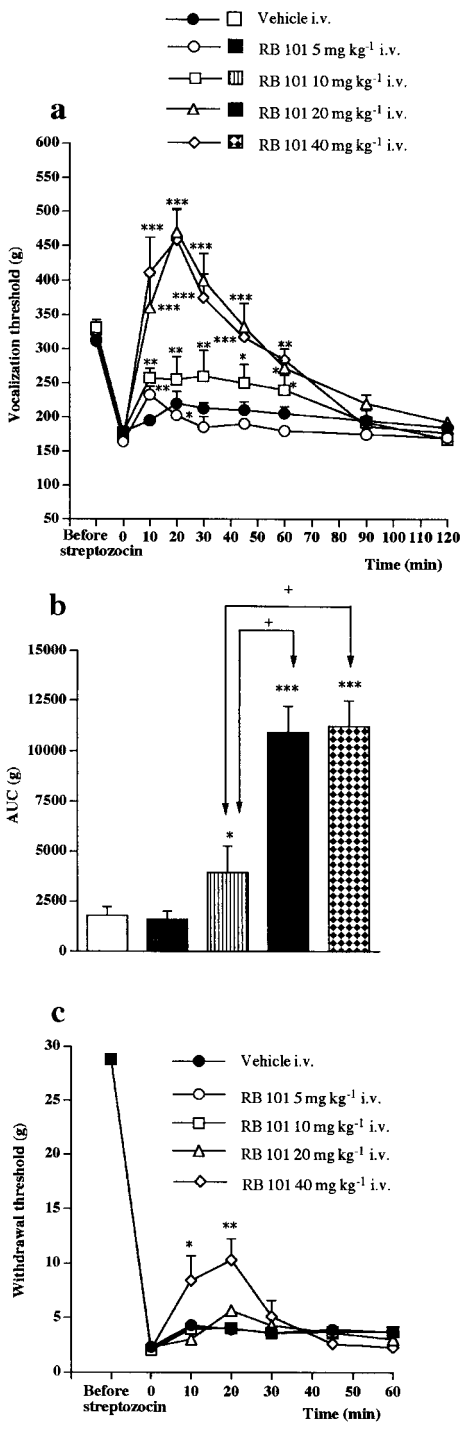

Figure 1 (a) Time course of the effect of intravenously administered vehicle or RB 101 (5, 10, 20 and 40 mg kg^{-1}) on the paw pressure-induced vocalization thresholds in diabetic rats. Vocalization thresholds determined before (0) and after drug injection are expressed as grams (g). Bars = + s.e.mean. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *versus* the corresponding predrug values. (b) Corresponding areas under the vocalization threshold-time curves (AUC) were obtained by joining the mean values of the vocalization thresholds (grams) between 0 and 120 min. $^{*}P<0.05$, $^{***}P<0.001$ *versus* the scores obtained after vehicle, $+P<0.05$ between the different doses. (c) Time course of the effect of intravenously administered vehicle or RB 101 (10, 20 and 40 mg kg^{-1}) on the withdrawal thresholds in diabetic rats submitted to a mechanical innocuous stimulus (von Frey hair test). Withdrawal thresholds determined before (0) and after drug injection are expressed as grams (g). Bars = + s.e.mean. $^{*}P<0.05$, $^{**}P<0.01$ *versus* the corresponding predrug values.

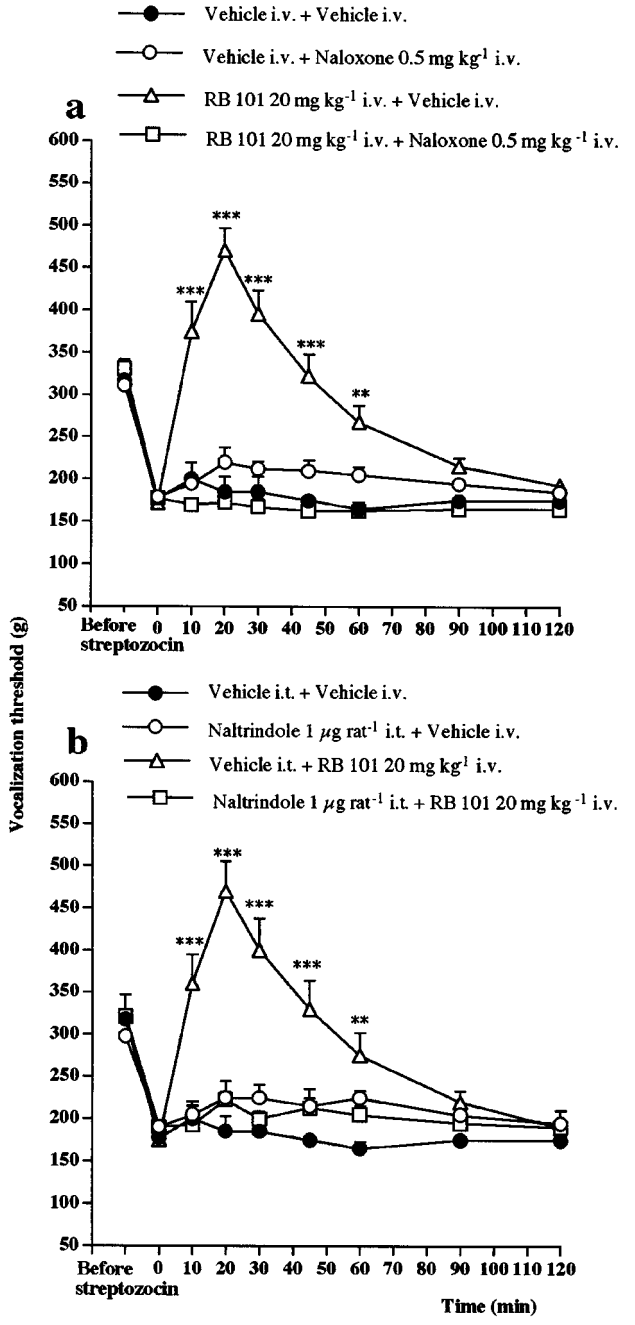

Figure 2 (a) Time course of the effect of vehicle i.v. + vehicle i.v., vehicle i.v. + naloxone 0.5 mg kg^{-1} i.v., RB 101 20 mg kg^{-1} i.v., + vehicle i.v., and RB 101 20 mg kg^{-1} i.v. + naloxone 0.5 mg kg^{-1} i.v., on the paw pressure-induced vocalization thresholds in diabetic rats. Vocalization thresholds determined before (0) and after drug injection are expressed as grams (g). Bars = + s.e.mean. $^{**}P<0.01$; $^{***}P<0.001$ *versus* the corresponding predrug values. (b) Time course of the effect of vehicle i.t. + vehicle i.v., naltrindole 1 μg rat^{-1} i.t. + vehicle i.v., vehicle i.t. + RB 101 20 mg kg^{-1} i.v., and naltrindole 1 μg rat^{-1} i.t. + RB 101 20 mg kg^{-1} i.v., on the paw pressure-induced vocalization thresholds in diabetic rats. Vocalization thresholds determined before (0) and after drug injection are expressed as grams (g). Bars = + s.e.mean. $^{**}P<0.01$; $^{***}P<0.001$ *versus* the corresponding predrug values.

induce a highly significant dose-related antinociceptive effect. These results are in complete agreement with those obtained in other chronic pain models as in mononeuropathic and

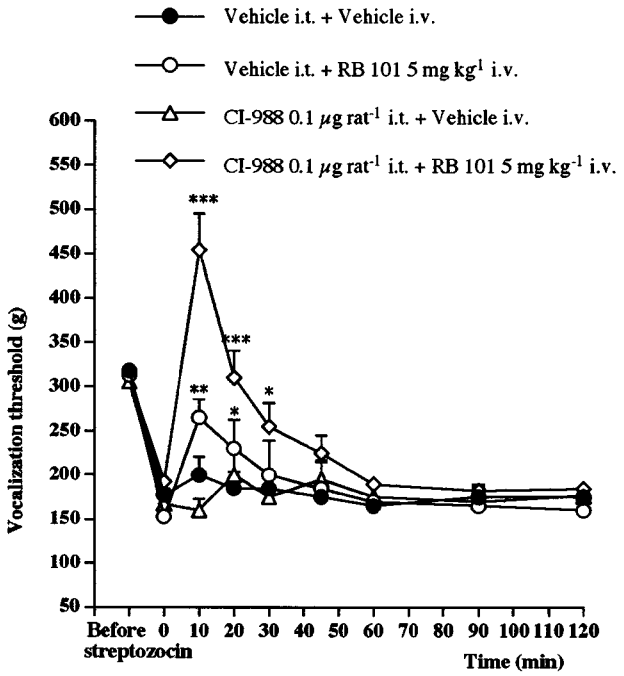

Figure 3 Time course of the effect of vehicle i.t. + vehicle i.v., vehicle i.t. + RB 101 5 mg kg^{-1} i.v., CI-988 0.1 μg rat^{-1} i.t. + vehicle i.v., and CI-988 0.1 μg rat^{-1} i.t. + RB 101 5 mg kg^{-1} i.v. on the paw pressure-induced vocalization thresholds in diabetic rats. Vocalization thresholds determined before (0) and after drug injection are expressed as grams (g). Bars = + s.e.mean. $^{*}P<0.05$, $^{**}P<0.01$; $^{***}P<0.001$ *versus* the corresponding predrug values.

arthritic rats (Attal *et al*., 1991; Perrot *et al*., 1993; Lee *et al*., 1994). However, the effects observed with the highest dose (40 mg kg^{-1}, i.v.) plateaued; this plateau corresponds very likely to the maximal existing levels of the endogenous opioid peptides which can be protected by the inhibitor. Similar results have been obtained using kelatorphan, another dual inhibitor of enkephalin inactivating enzymes (Roques *et al*., 1993), in mononeuropathic rats (Lee *et al*., 1994). It has been suggested that the analgesic potency of the peptidase inhibitors may directly depend on the importance of the extracellular release of endogenous peptides due to nociceptive stimuli (Lee *et al*., 1994). The main advantage of modifying the concentration of the endogenous peptides by using peptidase inhibitors is that the effects are only induced if the receptors are tonically or phasically stimulated by their natural mediators (review in Roques *et al*., 1993). Thus, these results show that by blocking enkephalin catabolism, hyperalgesia due to diabetes is strongly reduced suggesting an increased basal release of enkephalins in conditions of diabetes. Nevertheless, the maximal antinociceptive effect is obtained with 20 mg kg^{-1} and failed to raise the cut-off (460 g instead of 750 g). Whereas RB 101 was highly effective against mechanical hyperalgesia, it exerted a poor antiallodynic effect against tactile stimuli and failed to alleviate thermal allodynia. This difference of activity between: (i) hyperalgesia and allodynia: and (ii) tactile and thermal stimuli may be due to the level of integration of the response to the stimuli, i.e., supraspinal for vocalization (integrated response) and spinal for paw or tail withdrawal (reflex response). It is well known that morphine suppresses the vocalization response at a lower dose than that necessary to increase paw withdrawal threshold (Courteix *et al*., 1994; 1998), and the same scheme can be applied to endogenous opiates. It may also be due to the characteristics of the applied stimulus, the fibres involved in nociception or allodynia being not the same according to the intensity (innocuous or noxious) and to the nature (mechanical or thermal) of the stimulus. Actually, it is admitted that C-fibres are involved in the response to warm (42°C) stimulus, Aδ fibres in the response to mechanical stimulus and large Aβ (low threshold fibres) in the response to tactile stimulus. Allodynia being due to a change in the central processing of inputs from low threshold mechanoreceptor.

The involvement of the opioid receptors in the RB 101-induced antinociceptive effect was confirmed by the ability of naloxone (μ- and low δ-opioid receptor antagonist) and naltrindole (δ- and low μ-opioid receptor antagonist) to prevent this response. This indicates that the analgesic response triggered by RB 101 involves the endogenous opioid peptides that stimulate μ- and δ-opioid receptors in the structures involved in the control of the nociceptive stimuli (Noble *et al*., 1992) and confirm the specificity of RB 101. These latter authors have demonstrated that both the μ- and the δ-opioid receptors are involved in the tail flick response and in the motor response to tail electric stimulation and that only the μ-opioid receptors are involved in the hot plate and writhing tests (Roques *et al*., 1993).

Our study has also shown that coadministration of an inactive dose of the CCK-B receptor antagonist, CI-988, and of the lowest effective dose of RB 101 strongly increased vocalization threshold in the diabetic rats. Our results are reminiscent of previous studies, which provided evidence for spinal and supraspinal sites for the potentiation between opioids and CCK-B receptor antagonists (Noble *et al*., 1993; Ossipov *et al*., 1994; Pu *et al*., 1994). This effect is in complete agreement with the potentiation between morphine and the CCK-B receptor antagonists, largely documented through behavioural and electrophysiological experiments (Stanfa & Dickenson, 1993; Zhou *et al*., 1993; Xu *et al*., 1994; Chapman *et al*., 1995). Furthermore, CCK-B antagonists have been previously reported to facilitate several types of response induced by the endogenous enkephalins, such as the antinociceptive effects of RB 101 (Noble *et al*., 1993; Valverde *et al*., 1994), and the expression of noxiously evoked spinal c-Fos (Honoré *et al*., 1997). The potentiation of RB 101 effect by CI-988 was not due to an intrinsic antinociceptive response resulting from CCK-B receptor blockade, since CI-988 used alone at a dose of 0.1 μg per rat was unable to modify pain threshold (Coudoré-Civiale *et al*., 2000). Several hypotheses can be proposed: Thus, CCK could produce allosteric changes in the opioid receptors leading to a postreceptor modification which counter acts the opioid effector system (Valverde *et al*., 1994). On the other hand, the potentiation of endogenous enkephalin-induced antinociception by CCK-B antagonists could also occur directly by changes in events after receptor stimulation or blockade (review in Noble *et al*., 1999). Actually, CCK and opioid receptors, which both belong to the group of guanine nucleotide-binding protein-coupled binding sites (Wank *et al*., 1992) are colocalized on the same neurons in discrete areas of rat brain (Pohl *et al*., 1990), and an improvement of the intracellular transduction mechanisms associated with opioid

receptor stimulation could result from CCK-B receptor blockade (Valverde *et al*., 1994). Our results, which demonstrate a facilitating effect of CCK-B antagonists on the antinociceptive responses due to the endogenous enkephalins protected from their catabolism by RB 101, confirm the opposing physiological roles of endogenous CCK and opioid peptides in the control of pain and could have important clinical implications. Indeed, these results offer new insights into the controversies surrounding the utility of opioid analgesics in the treatment of neuropathic pain syndromes and suggest new pharmacological tools for neuropathic pain therapy.

To conclude, it can be underlined that inhibition of the enzymes implicated in the inactivation of the endogenous enkephalins is a useful tool to assess the physiological role of the endogenous opioid system. In addition, the use of RB 101 allowed a critical evaluation of potentiating the analgesic effects of the endogenous opioids without side effects of morphine in neuropathic pain. Moreover, coadministration of the inhibitors of enkephalin catabolism with the CCK-B antagonists appears to be a promising perspective in the management of pain using very low doses and inducing less side effects than opiates after chronic treatment.

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