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Depressant effect on a C-fibre reflex in the rat, of RB101, a dual inhibitor of enkephalin-degrading enzymes

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Abstract

The effect of *N*-[(*R*,*S*)-2-benzyl-3[(*S*)-(2-amino-4-methylthio)butyldithiol]-1-oxopropyl]-L-phenylalanine benzyl ester (RB101), a dual inhibitor of the enkephalin-degrading enzymes, neutral endopeptidase and aminopeptidase N, was assessed in anaesthetised rats on the C-fibre reflex elicited by electrical stimulation within the sural nerve territory and recorded from the ipsilateral biceps femoris muscle. The temporal evolution of the pharmacological response was monitored by the repeated application of a constant stimulus intensity, namely three times threshold (3 T). In addition, recruitment curves were built by varying the stimulus intensity from 0 to 7 T. RB101 (7.5, 15 and 30 mg kg^{-1}, i.v.) induced a dose-dependent, naloxone-reversible depression of the reflex, which lasted around 60 min with the highest dose. The ED_{50} was calculated as 16.9 mg kg^{-1}. Analyses of the recruitment curves revealed: (1) a significant increase of threshold; (2) a significant depression of the reflex in the ascending part of the curve; and (3) a lack of major depressive effects on the responses elicited by the strongest stimuli (corresponding to the plateau of the curve). The increase in the nociceptive threshold by enkephalin-degrading enzyme inhibitors, confirms previous data obtained from behavioural tests. In addition, the present study revealed an efficacy of these compounds over a wide range of stimulus intensities, albeit excluding the highest.

Keywords: RB101; Neutral endopeptidase; Aminopeptidase N; Enkephalin; Analgesia; C-fibre reflex

1. Introduction

One possible way of reproducing the analgesic effects of morphinic drugs without their usual drawbacks, is to improve the potency of the physiological systems for pain control by preserving the endogenous opioid peptides, [Met^5]- and [Leu^5]enkephalins from degradation. Enkephalins are inactivated by two metallopeptidases, the neutral endopeptidase (neprilysin) and the aminopeptidase N (for review, see Roques et al., 1993). The distribution of these enzymes in the central nervous system largely overlaps the distribution of both the peptides themselves and their specific binding sites, the μ- and δ-opioid receptors (Waksman et al., 1986; Pollard et al., 1989; Back and Gorenstein, 1990; Konkoy and Davis, 1995; Noble et al., 2001). Inhibitors of these peptidases are expected to exert their effects when there is an effective secretion of enkephalins (Chipkin, 1986). That there is a tonic release of endogenous opioids in central structures related to pain control, was suggested by the hyperalgesia elicited in the rat by the opioid receptor antagonist, naloxone (Jacob and Ramabadran, 1981; Costentin et al., 1986). However, such an effect was not confirmed in healthy human beings (Boureau et al., 1978). Phasic release of enkephalins following noxious stimulation has been described in the spinal cord and mesencephalon of the rat (Yaksh and Elde, 1981; Cesselin et al., 1985; Bourgoin et al., 1990; Noguchi et al., 1992). In contrast, the involvement of enkephalins in other functional control systems appears to be weak since, at least under basal conditions, their concentrations in most regions of the brain remain too low to saturate opioid receptors, even when the peptides are protected from enzymatic degradation (Williams et al., 1987; Ruiz-Gayo et al., 1992). This probably accounts for the lack of major side effects of peptidase inhibitors (Noble et al., 1992a,b, 1993; Hutcheson et al., 2000).

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Because of the complementary roles of neutral endopeptidase and aminopeptidase N in enkephalin inactivation, it has been assumed that inhibition of the two enzymes is required to achieve clinically efficient analgesia. This resulted in the concept of dual inhibitors (Fournié-Zaluski et al., 1984) and the development of *N*-[(*R*,*S*)-2-benzyl-3[(*S*)-(2-amino-4-methylthio)butyldithiol]-1-oxopropyl]-L-phenylalanine benzyl ester (RB101). RB101 is a lipophilic 'prodrug' that associates a *N*-(mercaptoacyl)amino acid as the neutral endopeptidase inhibitor, and a β-mercaptoalkylamine as the aminopeptidase N inhibitor, through a disulphide bridge which is cleaved in the brain by a biologically dependent mechanism (Fournié-Zaluski et al., 1992; Noble et al., 1992b). The release of the active inhibitors in the CNS was confirmed in vivo, following systemic administration of RB101, by both the blockade of neutral endopeptidase activity and the extracellular increase of [Met^5]enkephalin-like material (Noble et al., 1992b; Daugé et al., 1996). The dose-dependent and naloxone-reversible effects of RB101 were established from several models of nociception using thermal, electrical or chemical stimulation (Noble et al., 1992b, 1995; Jayaram et al., 1997; Xu et al., 1997). Antinociceptive effects of dual inhibitors were also reported in both inflammatory (Perrot et al., 1993; Maldonado et al., 1994) and neuropathic (Lee et al., 1994; Coudore-Civiale et al., 2001) models of chronic pain. By comparison with morphine, equi-analgesic doses of RB101 induced significantly less respiratory depression (Jayaram et al., 1997; Boudinot et al., 2001). Furthermore, no major signs of dependence or tolerance to the antinociceptive effects were reported following chronic administration (Noble et al., 1992a,c, 1994a; Hutcheson et al., 2000).

Since clinical pain is rarely restricted to that occurring around the pain threshold, it is essential to obtain information regarding drug efficacy with suprathreshold stimuli. For this reason, it was important to further the study of RB101, by analysing its effects on responses elicited by a wide range of stimulus intensities in the rat, as had already been done with morphine (Strimbu-Gozariu et al., 1993; Guirimand et al., 1994, 1995a). We therefore recorded the electromyographic (EMG) reflex response elicited in the biceps femoris muscle, by electrical stimulation of cutaneous C-fibre afferents in the territory of the sural nerve (Falinower et al., 1994). This polysynaptic spinal reflex is largely controlled by supraspinal structures (Willis, 1982; Falinower et al., 1994). Such flexion reflexes provide an objective index for experimental pain, as was shown in humans by Willer (1977) for the R_{III} component of the reflex, where the thresholds for EMG responses and painful sensations are closely matched. The largely non-invasive nature of this method allows the examination of responses to a wide range of stimulus intensities. The method was shown to be a valid pharmacological test, by its use for the study of morphine (Strimbu-Gozariu et al., 1993; Guirimand et al., 1994, 1995a), sufentanil (Adam et al., 2001), buprenorphine (Guirimand et al., 1995a,b), clonidine (Gozariu et al., 1996) and non-steroidal anti-inflammatory drugs (Bustamante et al., 1996, 1997). In the present report, the effects of i.v. RB101 were analysed using two paradigms of stimulation: constant suprathreshold stimulation, namely at three times threshold intensity (3 T) and the use of stimuli of increasing intensities (1–7 T).

2. Methods

2.1. Animal preparation

The general procedures were essentially similar to those described previously (Strimbu-Gozariu et al., 1993). Experiments were carried out on 45 male Sprague–Dawley rats, weighing 300–350 g. During the surgical procedures, the animals were deeply anaesthetised with 3% halothane in 100% O_2. The trachea, a jugular vein and a carotid artery were cannulated. After surgery, the concentration of halothane was lowered to 1%. Throughout the experiments, the animals were artificially ventilated. Respiratory rate (55 counts/min), O_2, end-tidal CO_2 and halothane levels were monitored continuously using a capnometer (Capnomac II®, Datex Instruments, Helsinki, Finland). The measurements of CO_2 and halothane were performed by infrared absorption and the O_2 levels with a fast paramagnetic analyser. These parameters were displayed digitally and each was under the control of an alarm. The volume of ventilation was adjusted to maintain a steady acid–base equilibrium, as assessed by the levels of end-tidal CO_2 (between 3.2% and 3.7%). Heart rate and blood pressure were also monitored. Core temperature was maintained at 37 ± 0.5 °C by means of a homeothermic blanket system.

2.2. Electrophysiological techniques

The methods used have been described previously (Strimbu-Gozariu et al., 1993; Falinower et al., 1994; Guirimand et al., 1994). A pair of non-insulated platinum–iridium needle electrodes were inserted subcutaneously into the medial part of the fourth, and the lateral part of the fifth toe, within the receptive field of the sural nerve. Electrophysiological recordings were made from the ipsilateral biceps femoris muscle via another pair of non-insulated platinum–iridium needles, inserted 0.5 cm apart into the muscle.

The electrical stimuli consisted of single, square-wave shocks of 2-ms duration, delivered once every 6 s (0.17 Hz) from a constant current stimulator. The stimulus intensities and the EMG responses were fed to an oscilloscope for continuous monitoring and to a computerised system (PLS®, Notocord, Igny, France) for on-line digitisation. The digitised EMG responses were full-wave rectified. The C-fibre-evoked responses were integrated within a time window 125–575 ms after the stimulus onset (Fig. 1A). Base line EMG activity was also integrated over a 450-ms

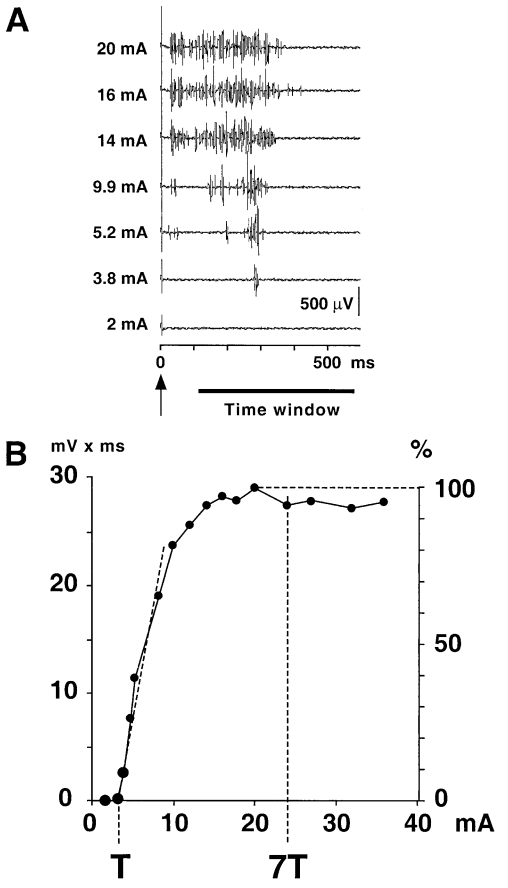

Fig. 1. Individual example of reflex responses elicited by several stimulus intensities. (A) Recruitment of the reflex with increasing stimulus intensities. The responses were elicited by electrical stimulation within the territory of the sural nerve at the time indicated by the arrow (2-ms duration; intensities indicated to the left of each recording). To analyse the C-fibre-evoked responses, the recordings were full-wave rectified and integrated within a 125- to 575-ms time window (horizontal bar). (B) Example of a recruitment curve. Abscissa: current intensity; ordinate: integrals of the C-fibre-evoked responses. The amplitude of the reflex increased monotonically as a function of stimulus intensity and reached a plateau at the highest levels of stimulation. The threshold of the C-fibre reflex was determined as the intersection of the dotted regression line and the abscissa. In the text and subsequent figures concerning the recruitment curves, current intensity is expressed in terms of multiples of the control threshold (T) and the integrals of the reflex responses in terms of percentages of the maximal control responses (upper horizontal line).

period, from 5500 to 5950 ms after the stimulus onset, i.e. just before the next stimulus. The temporal evolution of these integral values, expressed in mV ms, was visualised throughout the experiments. For further analyses, the individual values of the C-fibre reflex were corrected by subtraction of the related base line values.

2.3. Characteristics of the control period and general experimental procedure

During a preliminary phase, the characteristics of the reflex were determined. Twenty to thirty min after the end of the surgical preparation and decrease in the level of anaesthesia, a series of 15-mA stimuli was applied to the sural nerve territory. Stability of the C-fibre evoked responses was regarded as a prerequisite for starting the main experimental procedures.

After the stabilisation period, the control threshold intensity was assessed. A 'recruitment curve' was built by increasing the stimulus intensity (Fig. 1B). The magnitude of the C-fibre-evoked responses increased monotonically, and reached a plateau at high intensities. The threshold intensity was taken to be the intersection of the straight regression line with the abscissa. A constant stimulus current (3 T) was subsequently applied, which induced a response the magnitude of which was on the ascending part of the recruitment curve. During the first 15 min, the stability of this response was checked. Thereafter, 5 min before the injection of RB101 or the vehicle, the intensity of stimulation was gradually increased in order to produce at least 15 points to build the control recruitment curve. The stability of the threshold intensity was verified, before starting the pharmacological procedure.

2.4. Pharmacological procedure

The first series of experiments determined the overall effect of RB101 on the C-fibre reflex elicited by 3 T stimulation, its time-course and the dose–effect relationship. Three doses (7.5, 15 and 30 mg kg^{-1}, i.v.) were administered using different dilutions (37.5, 75 and 150 mg/10 ml, respectively) in order to inject the same volume of solution (0.2 ml/100 g of body weight). Control animals received the same volume of the vehicle. In the second series of experiments, the response to RB101 (30 mg kg^{-1}, i.v.) was analysed using both the constant stimulation and the recruitment curve paradigms. In these latter experiments, the effects of naloxone (0.4 mg kg^{-1}, i.v.) or vehicle were tested 20 min after the injection of RB101.

2.4.1. Analysis of the constant stimulation paradigm

The time-course of the effects was visualised by calculating the mean amplitude of 10 successive reflex responses within 1-min periods and expressing these as percentages of the control value. In each individual experiment, this control value was computed within a 2-min period, between 7 and 5 min before injection. Two-minute periods were also used to quantify the effects of the drug (14–16 min after RB101 or vehicle injection and 4–6 min after naloxone injection).

2.4.2. Analysis of the recruitment curve paradigm

Although at least 15 points were used to build the recruitment curves, only 9 points, namely those for 1, 1.5, 2, 2.5, 3, 4, 5, 6 and 7 T were considered in order to simplify the data processing. In some cases, when one of these intensities had not actually been applied during the experiment, the response was estimated by linear regression between the two nearest points. For comparison between pre- and post-drug recruitment curves, individual EMG

responses were expressed as percentages of the maximal response recorded during the control period, which was generally reached at an intensity of 4–7 T (Fig. 1B).

2.5. Statistical analyses

Dunnett's *t*-test was used to assess the significance of changes observed in the amplitude of the C-fibre reflex (3 T) with time after injection of RB101. Analyses of variance (ANOVA) were performed to compare the pre- and post-drug data within each group (vehicle- or RB101-injected rats), i.e. the amplitudes of the reflex in the constant stimulation and recruitment curve paradigms, and the threshold intensities. When this revealed a significant 'factor' effect, differences were evaluated separately using the Scheffé test. The *t*-test was used for comparisons between the two groups. Results were considered significant at $P < 0.05$.

Dose–response relationship was analysed using a least-square linear regression. One-way ANOVA was performed to test the linearity of the curve. Fieller's theorem was used to determine 95% confidence limits (95% CL) (Bolton, 1990).

2.6. Drugs

Racemic RB101 (mesylate salt) (Fournié-Zaluski et al., 1992) was dissolved in a vehicle consisting of: ethanol (10%), cremophor EL (10%) and distilled water (80%). Cremophor EL was obtained from Sigma (USA), and naloxone HCl (Narcan®) (0.4 mg ml^{-1} in saline) from Du Pont Merck Pharma (USA).

3. Results

3.1. Characteristics of the reflex in the control period

As previously described (Strimbu-Gozariu et al., 1993; Falinower et al., 1994; Guirimand et al., 1994, 1995a), electrical stimulation within the territory of the sural nerve elicited a two-component flexion reflex in the ipsilateral biceps femoris muscle (Fig. 1A). The first component, characterised by a short latency (~ 20 ms) and duration (~ 50 ms), and a low threshold (1.5–2.0 mA), resulted from the activation of myelinated fibres. The second component included two successive discharges (which progressively merged as the stimulus intensity was increased); this component had a longer latency (~ 150 ms at 3 T) and duration (~ 300 ms at 3 T), and a higher threshold (3.9 ± 0.3 mA) than the first component. It was elicited by the activation of unmyelinated (C-fibre) afferents (Falinower et al., 1994). This C-fibre reflex exhibited the classical electrophysiological features of a polysynaptic response, i.e. a decrease in latency and an increase in both duration and amplitude when the stimulus intensity was increased (Fig. 1A). Only this second component (in a 125- to 575-ms post-stimulus time window) was taken into account for the pharmacological studies.

3.2. Effects of RB101 in the constant stimulation paradigm

Individual examples of the temporal evolution of the C-fibre reflex elicited by the 3 T stimulus following RB 101 injections, are shown in Fig. 2. The lowest (7.5 mg kg^{-1}, i.v.) dose initially depressed, then slightly increased the responses; the total duration of this biphasic effect was less than 30 min. With higher (15 and 30 mg kg^{-1}) doses, only a depressant effect was seen; this increased with dose both in terms of amplitude and duration. Three min following 30 mg kg^{-1} RB101, the depression reached 85%, and was still present 60 min later. The mean results, which are shown in Fig. 3A, generally confirmed these individual observations. However, the biphasic effect described for the lowest dose was not found consistently (it occurred in only four of six cases); indeed the late facilitation did not reach statistical significance when data from the 6 experiments were pooled. Vehicle injections induced a transient but not significant depression of the reflex. The dose–response relationship was computed from the maximal depressant effect of RB101 (which occurred 3–5 min post-injection), after subtraction of the vehicle effect (Fig. 3B). An ED_{50} of 16.9 mg kg^{-1} (95% CL: 13.8–21.5 mg kg^{-1}) was calculated.

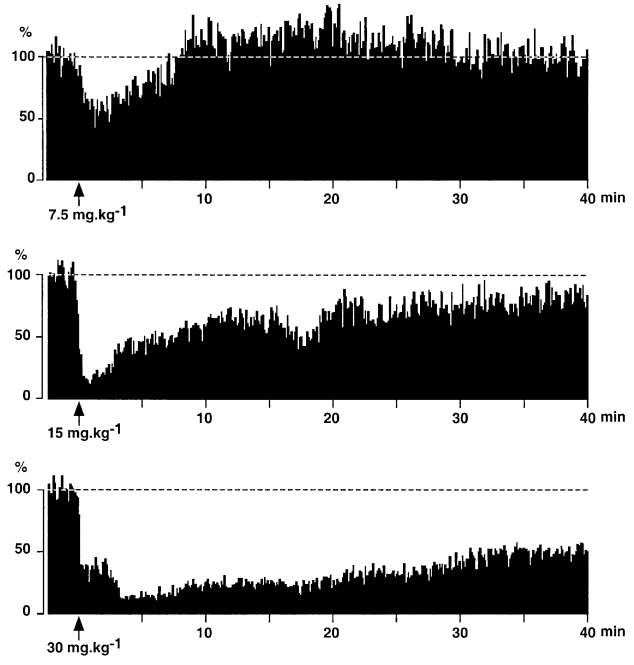

Fig. 2. Individual examples of responses to RB101 injections in the constant stimulation paradigm. The reflex was elicited by a stimulus delivered every 6 s, at an intensity of 3 T. The amplitude of the reflex is expressed as percentages of the mean control value measured before the RB101 injection.

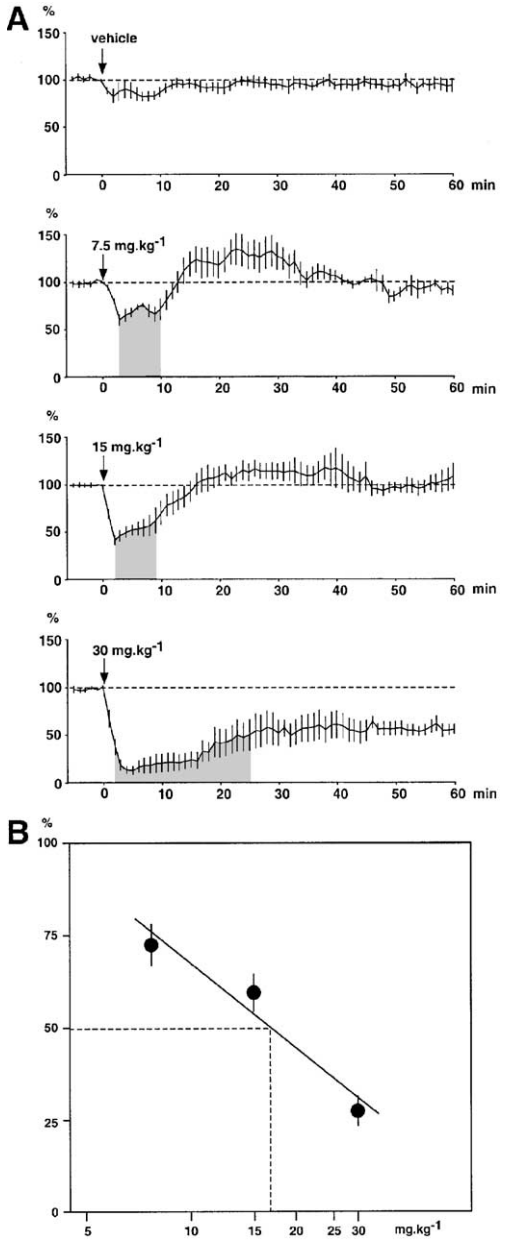

Fig. 3. Average responses to RB101 injections in the constant stimulation (3 T) paradigm. (A) Time course of the responses following RB101 injections: The mean amplitude (± S.E.M.) of the C-fibre reflex was calculated within 1-min periods, and expressed as percentages of the control value (number of experiments = 6, 6, 9 and 6 for the vehicle, and 7.5, 15 and 30 mg kg^{-1} doses of RB101, respectively). Grey areas indicate the periods where the reflex was significantly different from the controls (Dunnett's *t*-test; $P < 0.05$). (B) Dose–response relationship—abscissa: dose of RB101 on a logarithmic scale; ordinate: amplitude (± S.E.M.) of the C-fibre reflex, expressed as a percentage of the control value. The relationship is linear in the range of doses used ($r_{19} = -0.79$, ***$P < 0.001$). The dotted lines indicate the ED_{50} = 16.9 mg kg^{-1} [95% CL: 13.8–21.5 mg kg^{-1}].

Naloxone (0.4 mg kg^{-1}) completely reversed the depressant effect of 30 mg kg^{-1} RB101. The antagonism was significant when the results were compared to pre-injection values or to values obtained from animals not treated with naloxone, after an identical delay following the RB101 injection (Fig. 4A,B). Naloxone did not significantly modify EMG responses following injections of the vehicle.

3.3. Effects of RB101 in the recruitment curve paradigm and on threshold intensity

As previously described in rats (Duysens and Gybels, 1988; Falinower et al., 1994) and humans (Willer, 1985), the magnitudes of flexion reflexes increased with the intensity of electrical stimulation and then reached a plateau. Following 30 mg kg^{-1} RB101, the recruitment curve was shifted to the right, with the slope of the ascending part slightly but significantly, decreased (Fig. 5B,A). As compared to the control period or to vehicle-injected animals, the EMG responses were significantly depressed at stimulus intensities less than 5 T, corresponding to the ascending part of the curve. Naloxone (0.4 mg kg^{-1}) reversed these modifications (Fig. 5B).

RB101 (30 mg kg^{-1}) significantly increased the reflex threshold, which reached 7.3 ± 0.7 mA (cf. 3.8 ± 0.4 mA

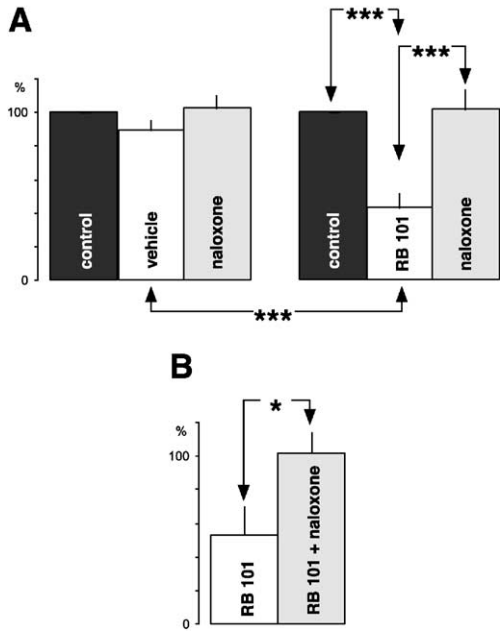

Fig. 4. Effects of RB101 and subsequent (20 min) injections of naloxone on the C-fibre-evoked responses, in the constant stimulation (3 T) paradigm. The amplitude (± S.E.M.) of the reflex is expressed as a percentage of the mean control value, measured 7–5 min before the injection of the vehicle or RB101. (A) The effects of the vehicle or RB101 (30 mg kg^{-1}) were measured 14–16 min after injection, those of naloxone (0.4 mg kg^{-1}) 4–6 min after injection. One-way ANOVA was used for comparison within each group: Vehicle-injected rats ($n = 7$): $F(2,18) = 1.637$ ($P = 0.22$); RB101-injected rats ($n = 11$): $F(2,30) = 16.714$ ($P < 0.001$). The Scheffé test was used for multiple comparisons, and the *t*-test for comparison between vehicle- and RB101-injected rats. Probability level: *** $P < 0.001$. (B) The responses were compared in rats receiving RB101 alone ($n = 6$) with rats receiving RB101 then naloxone ($n = 11$). In both cases, the measures were performed 24–26 min following the RB101 injection. The *t*-test was used for comparison (* $P < 0.05$).

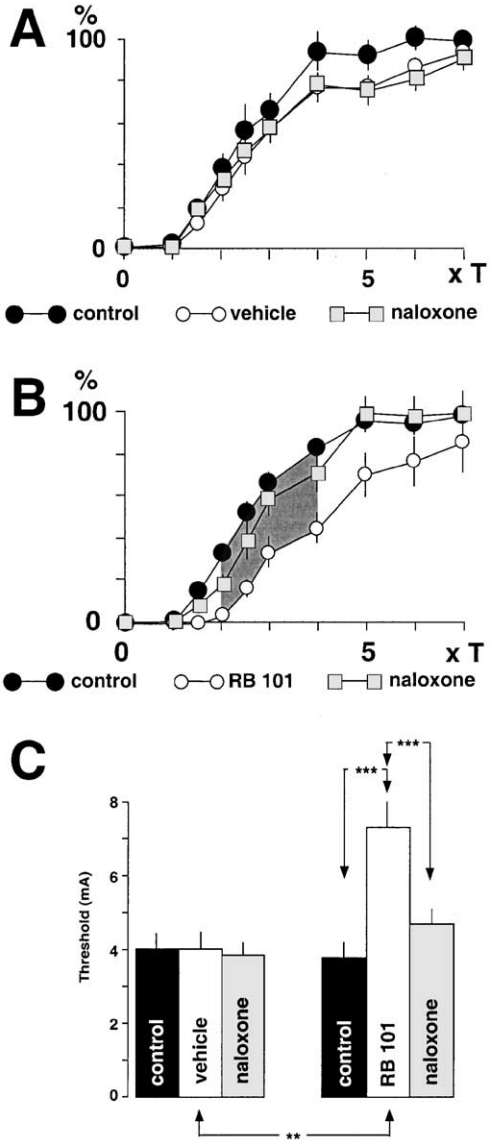

Fig. 5. Effects of RB101 and subsequent (20 min) injections of naloxone on the C-fibre reflex, within a wide range of stimulus intensities. (A, B) Abscissa: stimulus intensity, expressed as a multiple of the control threshold value (T); ordinate: amplitudes (±S.E.M.) of the EMG responses, expressed as percentages of the maximal value reached in the control recruitment curve. Recruitment curves were established before the injection of vehicle (A) or RB101 (30 mg kg^{-1}) (B), 17–19 min after injection, and 10–12 min after naloxone (0.4 mg kg^{-1}). RB101 slightly decreased the slope of the ascending part of the curve (* $P<0.05$). Differences between treatments were significant when the stimulus intensity did not exceed 4 T, as indicated by the grey area (One-way ANOVA and Scheffé tests: RB 101 vs. control $P<0.01$; RB 101 vs. naloxone: $P<0.05$; RB 101 vs. vehicle: $P<0.05$). (C) Thresholds (expressed in mA) computed from recruitment curves. Two-way ANOVA was used for comparison within each group. Vehicle-injected rats ($n=7$): $F(2,6)=0.876$ ($P=0.44$); RB101-injected rats ($n=11$): $F(2,10)=35.464$ ($P<0.001$). The Scheffé test was used for multiple comparisons, and the t-test for comparison between vehicle- and RB101-injected rats. Probability levels: ***$P<0.001$, **$P<0.01$.

during the control period and 4.0 ± 0.5 mA in vehicle-injected animals). Again, 0.4 mg kg^{-1} naloxone reversed these effects (Fig. 5C).

4. Discussion

A dose-dependent, naloxone-reversible depressant effect of RB101 was observed on a C-fibre reflex recorded in the rat. This effect was assessed using two paradigms of stimulation: (1) constant suprathreshold stimulation to follow the time-course of the pharmacological effect and (2) increasing stimulus intensities to build recruitment curves.

4.1. Constant stimulation paradigm

In the constant stimulation paradigm, the stimulus intensity was fixed at three times the threshold for the recruitment of the C-fibre reflex during the control period. The magnitudes of the corresponding responses were situated approximately in the middle of the ascending part of the recruitment curve. Three min after injection of 30 mg kg^{-1} RB101, the reflex was almost completely blocked, an effect that corresponded approximately to that observed with 4–6 mg kg^{-1} morphine (Guirimand et al., 1995a). However, the effect of RB101 was of shorter duration.

When computed from the maximal depressant effect of the drug, the ED_{50} for RB101 was 16.9 mg kg^{-1} (cf. 3.8 mg kg^{-1} for morphine; Guirimand et al., 1995a). A higher ED_{50} (25.3 mg kg^{-1}) has been reported with an equivalent test in acute spinalised, unanaesthetised rats (Xu et al., 1997). The possible implications of this difference in sensitivity between normal and spinalised animals, for the site and mechanism of action of RB101, are discussed below.

Although speculative, comparison of our data with others obtained from behavioural tests, reveals differences between the effective doses of RB101. In the hot-plate test in the mouse, a significant antinociceptive effect appeared at 5 mg kg^{-1}, which is close to the liminal dose in the present study. In the tail-flick test in the rat, a dose of 20 mg kg^{-1} was required. In tests using electrical stimulation of the rat tail, doses higher than 20 mg kg^{-1} were required to inhibit the withdrawal reflex, although 10 mg kg^{-1} significantly depressed more integrated responses such as post-stimulus vocalisation (Noble et al., 1992b). One may conclude that, depending on which test is used, there may be involvement of different central pain-processing structures and corresponding differences in the magnitude of the phasic release of enkephalin; these factors may produce the different sensitivities to the peptidase inhibitor.

Following the initial depression, the lowest (7.5 mg kg^{-1}) dose of RB101 induced a facilitation of the C-fibre reflex in some experiments. Such increase never appeared before 15–20 min, probably following partial elimination of this short-acting drug. This ‘pronociceptive’ effect is reminiscent of the hyperalgesia in the hot-plate test in mice, observed with doses of RB101 as low as 0.25–1 mg kg^{-1} (Noble et al., 1994b). A facilitation of the C-fibre reflex was

also reported following injections of morphine at doses less than 2 mg kg^{-1}, i.v. (Guirimand et al., 1995a).

4.2. Recruitment curve paradigm

Following 30 mg kg^{-1} RB101, the main modification observed in the stimulus–response curve was a shift to the right. In addition the slope was reduced slightly, albeit significantly. Such changes reflect: (1) an increase of threshold; (2) a significant depression of EMG responses in the ascending part of the curve; and (3) a lack of major depressant effect on the responses elicited by the strongest stimuli (corresponding to the plateau of the curve).

The reflex threshold increased by about 80%. A roughly similar increase was observed following 4 mg kg^{-1} morphine (Guirimand et al., 1995a). This rise in threshold intensity agrees with several earlier reports of the effects of peptidase inhibitors on behavioural responses (Roques et al., 1980; Kayser et al., 1989; Oshita et al., 1990; Schmidt et al., 1991; Noble et al., 1992b; Perrot et al., 1993; Jayaram et al., 1997; Chen et al., 1998). However, the relative contribution of Aδ- and C-afferent fibres in these tests is not known (Le Bars et al., 2001).

Not only was the decrease of the slope of the curve elicited by 30 mg kg^{-1} RB101 weaker than that seen following 4 mg kg^{-1} morphine (Guirimand et al., 1995a), but the depressant effect was not significant at the higher stimulus intensities (≥ 5 T). Both the minimal reduction of the 'gain' of the stimulus–response function and the lack of significant effect on supramaximal EMG responses are reminiscent of the 'ceiling effect', previously described for mixed inhibitors such as kelatorphan and PC12 (Kayser et al., 1989; Perrot et al., 1993; Lee et al., 1994; Tölle et al., 1994; Chen et al., 1998). Such a limitation is consistent with the hypothesis that the analgesic potency of peptidase inhibitors depends directly on the magnitude of the extracellular opioid release. As assessed by the displacement of bound [^{3}H]diprenorphine in the mouse brain (Ruiz-Gayo et al., 1992), the maximal occupation of opioid receptors by enkephalins was reached with 10 mg kg^{-1} RB101 and did not exceed 30%. In spite of the higher intrinsic activity of enkephalins as compared to morphine (Noble and Roques, 1995), both this observation and our present data suggest that the local increase in enkephalin concentration is too low to efficiently depress the transmission of nociceptive messages elicited by high stimulus intensities. In contrast, a saturation of the opioid binding sites may be achieved with adequate doses of morphine. Morphine is efficacious on the C-fibre reflex over the whole range of stimulus intensities (Guirimand et al., 1995a).

4.3. Hypothesis about the sites of action of RB101

As electrical stimulation bypasses nociceptor endings, we can exclude the involvement of a peripheral mechanism for the effects described herein. Evidence for peripheral mechanisms was obtained from studies with natural stimuli in models of inflammatory pain, where the local release of enkephalins is obvious (Perrot et al., 1993; Maldonado et al., 1994; Carlton and Coggeshall, 1997).

As for direct opioid receptor agonists, both spinal and supraspinal mechanisms have been proposed to explain the antinociceptive effect of peptidase inhibitors. High densities of both enkephalins and neutral endopeptidase have been described in the superficial laminae of dorsal horn (Waksman et al., 1986; Yaksh, 1987; Pretel and Piekut, 1991). Enkephalins, whether released from interneurones or descending fibres, inhibit nociceptive transmission via pre- and/or post-synaptic mechanisms (Hökfelt et al., 1977; Gibson et al., 1981; Ribeiro-da-Silva and Cuello, 1995). The direct spinal effect of peptidase inhibitors such as kelatorphan or RB101 has been clearly demonstrated by: (1) the increase in the spinal release of [Met^5]enkephalin following intrathecal application (Bourgoin et al., 1986); (2) the depression of nociceptive responses of dorsal horn neurones following intrathecal application or electrophoretic ejection in the substantia gelatinosa (Dickenson et al., 1986, 1987; Morton et al., 1987); (3) the increase in the threshold for behavioural responses, elicited by intrathecal injection (Oshita et al., 1990); (4) the dose-dependent depression of the C-fibre reflex in the spinalised, unanaesthetised rat (Xu et al., 1997).

The role of a supraspinal component in the antinociceptive effect of peptidase inhibitors is suggested by the high concentration of both endogenous opioids and neutral endopeptidase-positive cell bodies in the brainstem nuclei involved in the ascending and descending circuitry of pain processing (Waksman et al., 1986; Back and Gorenstein, 1990). Dual inhibitors increase brain levels of endogenous enkephalins at both spinal and brain levels (Bourgoin et al., 1986; Daugé et al. 1996). Moreover, in the hot-plate test in the mouse, the intracerebroventricular (i.c.v.) injection of RB101 induced a dose-dependent, naloxone-reversible analgesia, while the i.c.v. administration of the μ-opioid receptor antagonist, β-fulnaltrexamine, partially depressed the effects of systemic RB101 (Noble et al., 1992b; Noble and Roques, 1995). The supraspinal structures involved were specified by microinjection of the opioid receptor antagonist, methylnaloxonium, into the periaqueductal grey and the nucleus raphe magnus. Injections in both significantly reduced the analgesic effect of systemic RB101, as evaluated with the tests using electrical stimulation of the rat tail (Valverde et al., 1996). In the C-fibre reflex model, a contribution of a supraspinal component in the response to i.v. RB101 is possible, as suggested by the lower values of ED_{50} obtained in intact as opposed to spinalised rats (Xu et al., 1997) (v. supra); however, differences in experimental protocols may also contribute to the differences between doses.

The increase in the C-fibre reflex, induced by low doses of RB101 and also seen with morphine, might be relevant to several mechanisms. Low synaptic concentrations of exogenous or endogenous opioid agonists may facilitate the

transmission of nociceptive inputs at the spinal level. A facilitation of the C-fibre reflex has already been shown following intrathecal or systemic injections of morphine or RB101 (Wiesenfeld-Hallin et al., 1991; Guirimand et al., 1995a; Xu et al., 1997). Such effects were attributed to a preferential involvement of presynaptic opioid receptors, by low opioid concentrations, or to an excitatory opioid modulation of sensory afferents, resulting in both cases, in a paradoxical increase in transmitter release from primary afferent terminals (Starke et al., 1989; Crain and Shen, 1990). The facilitation of the C-fibre reflex after low doses of RB101 could also be explained by a supraspinal action, involving brainstem areas that modulate the activity of descending pathways. Microinjection of morphine in the ventrolateral periaqueductal grey produces both facilitation and inhibition of nociception (Fang and Proudfit, 1998). Similar bi-directional effects were observed following microinjection in the dorsolateral pontine tegmentum, near the A7 cell group that provides the major noradrenergic innervation of laminae I–IV in the spinal cord; the facilitation predominated when low doses were injected, and was blocked by intrathecal injection of α_1-adrenoceptor antagonists (Holden et al., 1999). Since in addition, Met-enkephalinergic neurones were evidenced in the ventrolateral and ventromedial periaqueductal grey, that project to the dorsolateral pontine tegmentum (Holden and Proudfit, 1994, 1998), the hyperalgesic response to low doses of i.v. RB101 might well involve the α_1-noradrenergic descending pathway.

In conclusion, the present work confirms the antinociceptive effect of RB101, which was previously found with behavioural tests in which there was a significant increase in the threshold intensity for eliciting nociceptive responses. A significant reduction in the C-fibre reflex magnitude was observed only within a range of suprathreshold stimulus intensities and disappeared with supramaximal stimuli. The inhibition of enkephalin-degrading enzymes might induce analgesia comparable to that obtained with opioid receptor agonists such as morphine, except for the highest pain intensities.

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