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Weak tolerance to the antinociceptive effect induced by the association of a peptidase inhibitor and a CCK_B receptor antagonist

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Abstract

We have recently shown that CCK_B receptor antagonists such as PD-134,308, 4-{[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[tricyclo[3.3.1.1]dec-2-yloxy)carbonyl]amino]propyl]amino]-1-phenylethyl]amino}-4-oxo[*R*-(*R**,*R**)]-butanoate-*N*-methyl-D-glucamine, are able to strongly potentiate antinociception induced by endogenous enkephalins, protected from degrading enzymes by the mixed inhibitor RB 101, *N*-{(*R*,*S*)-2-benzyl-3[(*S*)-(2-amino-4-methylthio)butyldithio]-1-oxopropyl}-L-phenylalanine benzyl ester, at both spinal and supraspinal levels. In this study, the duration of this facilitatory response and the possible development of tolerance to this synergistic effect were investigated in the rat tail-flick test after acute and chronic treatment with PD-134,308 and RB 101. PD-134,308 facilitated and prolonged the antinociceptive responses induced by RB 101 (20 mg/kg, i.v.). The duration of the effect induced by PD-134,308 was also investigated by injecting this compound at different times before RB 101 administration. In the case of the tail-flick test, the improvement of RB 101 antinociceptive response was still significant 6 h after PD-134,308 (3 mg/kg, i.p.), whereas in the hot-plate test, this enhancement was only effective for 3 h after CCK_B receptor antagonist administration. In the case of a repeated administration of RB 101, the potentiation induced by PD-134,308 on the antinociceptive effect produced by the first injection of RB 101 (20 mg/kg, i.v.), was found almost identical after a second administration of RB 101 performed 190 min later. Chronic administration of RB 101 (20 mg/kg, i.v.) plus PD-134,308 (3 mg/kg, i.p.) administered for 5 days both once or twice per day, did not induce the development of tolerance to antinociception at the peak effect time. However, a decrease in the duration of the antinociceptive response was observed. These results indicate that the potent and long-lasting antinociceptive response induced by the coadministration of the peptidase inhibitor and the CCK_B receptor antagonist could have interesting perspectives in the clinical treatment of pain.

Keywords: Enkephalin catabolism inhibitor; Enkephalin, endogenous; CCK (cholecystokinin); RB 101; PD-134,308; Tail-flick; Hot-plate; Antinociception; CCK_B receptor antagonist; Tolerance

1. Introduction

Cholecystokinin octapeptide (CCK-8) is a neuropeptide with a widespread distribution in the central nervous system (CNS) (Vanderhaeghen et al., 1975). Two types of CCK receptors have been pharmacologically identified and recently cloned: CCK_A receptor, abundant in peripheral tissues, and CCK_B receptor, the predominant form found in the brain (Moran et al., 1986; Kopin et al., 1992; Wank et al., 1992; Lee et al., 1993). CCK-8 interacts at nanomolar concentrations with both types of CCK receptors (Innis and Snyder, 1980).

Radioimmunoassay and immunohistochemical studies have shown that CCK-8 and the opioid peptides, enkephalins, have a similar distribution within many areas of the CNS (Gall et al., 1987; Pohl et al., 1990), and it has been shown that CCK behaves as an antagonist of opiate analgesia (Faris et al., 1983). Thus, the administration of CCK receptor antagonists or active immunization against CCK, enhanced the antinociception produced by exogenous opiates (Faris et al., 1984;

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Baber et al., 1989). The physiological relevance of this response was recently demonstrated by the potentiation of the antinociceptive effects induced by endogenous enkephalins following administration of CCK_B receptor antagonists (Maldonado et al., 1993; Valverde et al., 1994). In these experiments the extracellular level of enkephalins was increased as previously reported (Ruiz-Gayo et al., 1992) by administration of RB 101, *N*-[(*R*,*S*)-2-benzyl-3[(*S*)-(2-amino-4-methylthio)butyldithio]-1-oxopropyl]-L-phenylalanine benzyl ester, a complete inhibitor of enkephalin-catabolizing enzymes, which is able to cross the blood-brain barrier (Fournié-Zaluski et al., 1992). The potentiation induced by CCK receptor antagonists on exogenous and endogenous opioid-induced antinociception is preferentially mediated through the CCK_B receptor (Dourish et al., 1990; Hughes et al., 1990; Wiesenfeld-Hallin et al., 1990; Maldonado et al., 1993). Thus, we have reported that different CCK_B receptor antagonists (PD-134,308, L-365,260 and RB 211) but not CCK_A receptor antagonists were able to improve the antinociceptive effects of RB 101 (Maldonado et al., 1993; Valverde et al., 1994).

The facilitatory effect induced by CCK_B receptor antagonists on RB 101 antinociceptive responses was shown to be significantly higher than that resulting from morphine administration (Valverde et al., 1994). However, in this previous study, the response was investigated only at the time of maximal effect of both RB 101 and PD-134,308, providing no details about the duration of the response and the possible development of tolerance after chronic treatment. This information is important to evaluate the possible therapeutic interest of this association in the management of pain. In the present study, we have investigated the pharmacokinetic properties of the association of RB 101 and PD-134,308 which were coadministered at different time points with subsequent measurement of the antinociceptive responses. The participation of the spinal and/or supraspinal structures in the observed long duration of analgesic responses was assessed by using antinociceptive tests that exhibit a different preferential level of integration, such as tail-flick and hot-plate tests.

On the other hand, the administration of non-selective CCK receptor antagonists (proglumide and benzocript) (Watkins et al., 1984; Rovati et al., 1985) prevents the development of morphine tolerance. This effect seems also to be mediated through CCK_B receptors since it was induced by selective CCK_B receptor antagonists, such as L-365,260 and PD-134,308 (Dourish et al., 1990; Xu et al., 1992). Based on these data, experiments were performed to evaluate, under different conditions of repeated administration, the possible development of tolerance to the association of RB 101 and PD-134,308.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (Depré, France) ranging in weight from 200 to 220 g were used in this study. Animals were housed in groups (five rats per group) for at least 3 days before the experiments were started, and food and water were available ad libitum. Each animal was used only once.

2.2. Chemicals

RB 101, *N*-{(*R*,*S*)-2-benzyl-3[(*S*)-(2-amino-4-methylthio)butyl dithio]-1-oxopropyl}-L-phenylalanine benzyl ester (Fournié-Zaluski et al., 1992), PD-134,308, 4-{[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[tricyclo[3.3.1.1]-dec-2-yloxy)carbonyl]amino]propyl]amino]-1-phenylethyl]amino}-4-oxo[*R*-(*R**,*R**)]-butanoate *N*-methyl-D-glucamine (Hughes et al., 1990) and L-365,260, (3*R*-(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-^{1}H-1,4-benzodiazepin-3-yl-*N*^{1}-(3-methylphenyl urea) (Chang and Lotti, 1986) were synthesized in our laboratory. Cremophor EL was purchased from Sigma Chemical (France). RB 101 was dissolved in the following vehicle: ethanol (10%), cremophor EL (10%) and distilled water (80%). PD-134,308 and L-365,260 were prepared in an aqueous suspension with carboxymethylcellulose (0.5%). All drugs were administered in a volume of 0.1 ml per 100 g of body weight. RB 101 was slowly injected i.v. at the dose of 20 mg/kg. PD-134,308 (1 or 3 mg/kg) and L-365,260 (0.5 mg/kg) were administered i.p. and s.c. respectively.

2.3. Antinociceptive tests

Radiant heat tail-flick in rats

The antinociceptive responses were determined by measuring the time required to respond to a painful radiating thermal stimulus, according to the method of D'Amour and Smith (1941). The rat was restrained so that the radiant heat source was focused onto the base of the tail. An automated tail-flick analgesymeter (Apelex, France) was used. The cut-off time was set at 15 s. For each rat, three determinations were carried out before drug injection (control latency). Antinociceptive responses were measured at different times after i.v. injection. The antinociceptive responses were expressed as a percentage of analgesia calculated by: percentage of analgesia = [(test latency − control latency)/(cut-off time − control latency)] × 100. The intensity of the thermal stimulus was previously adjusted to obtain a control latency between 4 and 6 s.

Hot-plate in mice

The test was based on that described by Eddy and Leimbach (1953). A glass cylinder (16 cm high, 16 cm

diameter) was used to keep the mouse on the heated surface of the plate, which was kept at a temperature of 55 ± 0.5°C using a thermoregulated water-circulating pump. The latency period until the mouse jumped was registered by means of a stopwatch (cut-off time 240 s). The responses were expressed as a percentage of analgesia using the following equation: percentage of analgesia = [(test latency − control latency)/(cutt-off time − control latency)] × 100.

2.4. Experimental schedule

First experiment

Animals were coadministered with PD-134,308 (3 mg/kg, i.p.) and RB 101 (20 mg/kg, i.v.) (experiment 1a) or with L-365,260 (0.5 mg/kg, s.c.) plus RB 101 (experiment 1b). The CCK_B receptor antagonists and the peptidase inhibitor were given 30 and 10 min, respectively, before the first tail-flick determination. Six tail-flick latencies were evaluated 10, 40, 70, 100, 130 and 190 min after RB 101 injection.

Second and third experiments

Tail-flick test in rats. RB 101 (20 mg/kg, i.v.) was given 10 min before first tail-flick determination. Two doses of PD-134,308 (1 and 3 mg/kg, i.p.) were used in order to evaluate if the duration of the antinociceptive effect was dose-related. PD-134,308 was injected at different times: 30 min (Fig. 2a and Fig. 3a), 1 h (Fig. 2b and Fig. 3b), 3 h (only with the lowest dose of PD-134,308, Fig. 3c), 6 h (Fig. 2c,d), and 9 h (only with the highest dose of PD-134,308, Fig. 2d) before the first tail-flick measurement. Three tail-flick determinations were carried out in all the cases, 10, 25 and 40 min after RB 101 injection.

Hot-plate test in mice. Mice were injected with RB 101 (20 mg/kg, i.v.) 10 min before the hot-plate determination. PD-134,308 was injected at different times: 30 min, 1 h, 3 h and 6 h before the test.

Fourth experiment

The effect of PD-134,308 on the antinociceptive response induced by repeated injections of RB 101 was investigated. PD-134,308 (3 mg/kg, i.p.) was injected 30 min before the first tail-flick determination. RB 101 (20 mg/kg, i.v.) was administered twice; 10 min before and 190 min after the first tail-flick measurement. Latencies were evaluated at 10 different time points: 10, 100 and 180 min after the first RB 101 administration, and 10, 25, 40, 70, 100, 130 and 190 min after the second RB 101 administration.

Fifth and sixth experiments

The possible development of tolerance to the antinociceptive responses induced during chronic treatment with the association of RB 101 and PD-134,308 was evaluated. In order to facilitate the chronic i.v. administration, rats were anesthetised by an i.p. injection of chloral hydrate (300 mg/kg), and the left jugular vein was cannulated with a polyethylene catheter (outer diameter = 0.96 mm, internal diameter = 0.58 mm), as previously described (Caine et al., 1993). The end of the catheter was passed out of the dorsal neck and secured to the skin with a suture. After the surgical procedure, rats were placed into individuals cages and a period of 4 days of post-surgical recovery was established before the start of the chronic treatment. RB 101 (20 mg/kg) was injected through the i.v. catheter and PD-134,308 (3 mg/kg) by i.p. route. Both compounds were administered once (5th experiment) or twice (6th experiment) daily during 5 days. Heparine was added (50 units/ml) to all i.v. solutions in order to overcome blood coagulation. RB 101 and PD-134,308 were administered each day at the same hour, 10 and 30 min, respectively, before the first tail-flick determination. Test were performed on the 1st, 3rd and 5th days of chronic treatment. Three tail-flick determinations were measured in all cases 10, 25 and 40 min after RB 101 injection.

2.5. Analysis of data

In the hot-plate study, group comparisons were made using a one-way ANOVA. Data concerning the tail-flick were analyzed by using a two-way analysis of variance (ANOVA) with repeated measures. The factors of variation were treatment (between subjects) and time (within subjects). In the tolerance study, group comparisons were made using a three-way ANOVA with repeated measures. The factors of variation were treatment (between subjects), day (within subjects) and time (within subjects, corresponding to the three determinations performed each day). Subsequent two-way ANOVA comparisons were performed in this experiment. In all the experiments, post-hoc individual dose effects were analyzed using Scheffé F-test comparisons following significant main effects of treatment by one-way ANOVA. The level of significance was $P < 0.05$.

3. Results

3.1. Enhancement of RB 101 antinociceptive responses by PD-134,308 or by L-365,260: pharmacokinetic study during 3 h (1st experiment)

Enhancement of RB 101 antinociceptive responses by PD-134,308

Two-way ANOVA revealed a significant treatment effect ($F(3,28) = 150.76$, $P < 0.0001$), time effect ($F(5,140) = 243.08$, $P < 0.0001$), and interaction be-

tween treatment and time ($F(15,140) = 108.00$, $P < 0.0001$). The facilitatory effect induced by PD-134,308 (3 mg/kg) on RB 101 (20 mg/kg) antinociceptive responses was significant during a long-lasting time period. Thus, one-way ANOVA revealed an effect of the treatment 10 ($F(3,28) = 674.71$, $P < 0.0001$), 40 ($F(3,28) = 109.23$, $P < 0.0001$), 70 ($F(3,28) = 16.58$, $P < 0.0001$), 100 ($F(3,28) = 11.01$, $P < 0.001$), 130 ($F(3,28) = 8.09$, $P < 0.001$), and 190 min ($F(3,28) = 5.99$, $P < 0.01$) after RB 101 injection. Subsequent individual means comparisons indicated that PD-134,308 potentiated the antinociceptive response induced 10, 40, 70, 100, and 130 min after RB 101 administration (Fig. 1a).

Enhancement of RB 101 antinociceptive responses by L-365,260

Two-way ANOVA showed a significant treatment effect ($F(3,28) = 21.42$, $P < 0.0001$), time effect $F(5,140) = 20.14$, $P < 0.0001$) and interaction between treatment and time ($F(15,140) = 35.38$, $P < 0.0001$). The facilitatory effect induced by L-365,260 (0.5 mg/kg) on RB 101 (20 mg/kg) antinociception was shorter than that induced by PD-134,308. One-way ANOVA showed an effect 10 ($F(3,28) = 110.15$, $P < 0.001$), 40 ($F(3,28) = 18.00$, $P < 0.001$), and 70 min ($F(3,28) = 7.43$, $P < 0.001$) after RB 101 (20 mg/kg) injection. Subsequent individual means comparisons indicated that L-365,260 (0.5 mg/kg) potentiated the antinociceptive response induced 10 and 40 min after RB 101 administration (Fig. 1b).

3.2. Effects induced on RB 101 antinociceptive response by the pre-treatment with PD-134,308 at different times in the tail-flick test in rats and in the hot-plate test in mice

Pharmacokinetic study during 9 h: tail-flick test in rats (second experiment)

PD-134,308 (3 mg / kg) administered 30 min before first tail-flick determination significantly enhanced RB 101 (20 mg / kg) antinociceptive response. Two-way ANOVA revealed a significant treatment effect ($F(3,28) = 303.07$, $P < 0.0001$), time effect ($F(2,56) = 199.79$, $P < 0.0001$), and interaction between treatment and time ($F(6,56) = 82.07$, $P < 0.0001$). One-way ANOVA showed an effect of the treatment 10 ($F(3,28) = 320.21$, $P < 0.001$), 25 ($F(3,28) = 150.08$, $P < 0.001$), and 40 min ($F(3,28) = 36.05$, $P < 0.001$) after RB 101 administration. Individual means comparisons indicated that PD-134,308 significantly potentiated the antinociceptive effect induced 10, 25 and 40 min after RB 101 injection (Fig. 2a).

PD-134,308 (3 mg / kg) administered 1 h before first tail-flick determination significantly enhanced RB 101 (20 mg / kg) antinociception. Two-way ANOVA revealed a significant treatment effect ($F(3,28) = 143.31$, $P < 0.0001$), time effect ($F(2,56) = 118.58$, $P < 0.0001$), and interaction between treatment and time ($F(6,56) = 37.12$, $P < 0.0001$). One-way ANOVA revealed an effect of the treatment 10 ($F(3,28) = 137.47$, $P < 0.001$), 25 ($F(3,28) = 72.50$, $P < 0.001$), and 40 min ($F(3,28) = 10.80$, $P < 0.001$) after RB 101. Subsequent individual

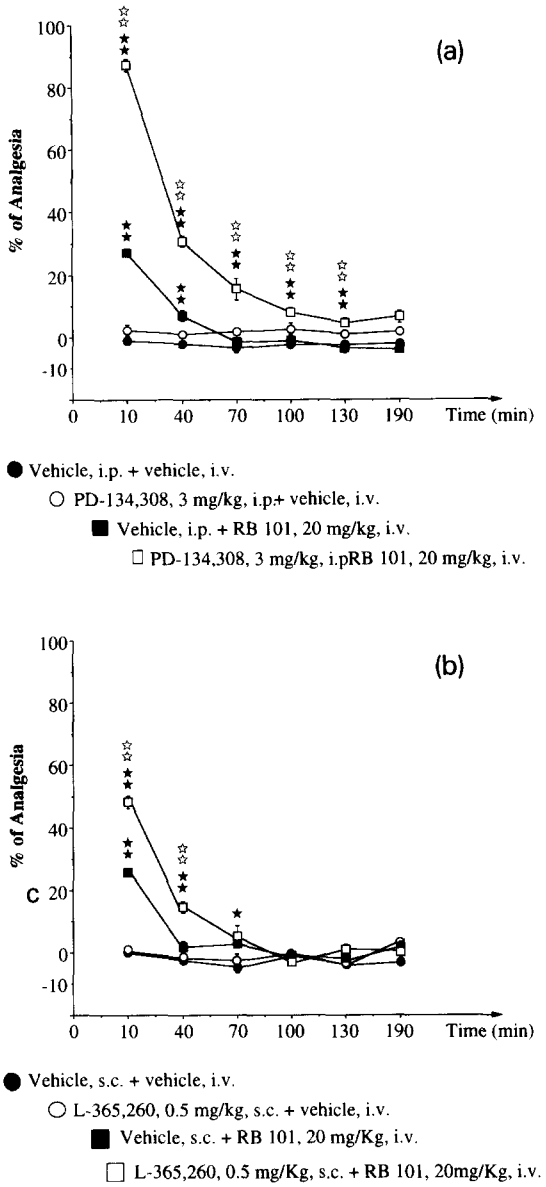


Fig. 1. Effects induced by PD-134,308 (a) and L-365,260 (b) on the antinociceptive responses induced by RB 101 in the rat tail-flick. PD-134,308 (3 mg/kg, i.p.) and L-365,260 (0.5 mg/kg, s.c.) were administered 30 min before the first tail-flick determination. RB 101 (20 mg/kg, i.v.) was injected 10 min before the first tail-flick measurement. Tail-flick latencies were tested at six different times: 10, 40, 70, 100, 130, and 190 min after RB 101 injection. The results are expressed as percentages of analgesia ± S.E.M. ($n = 8$ for each group). ★ $P < 0.05$; ★★ $P < 0.01$ vs. vehicle group, ☆☆ $P < 0.01$ vs. RB 101 (20 mg/kg) group (Scheffé's F-test).

means comparisons indicated that the responses induced 10, 25 and 40 min after RB 101 administration were potentiated by PD-134,308 (Fig. 2b).

PD134,308 (3 mg/kg) administered 6 h before first tail-flick determination induced a slight but still significant enhancement on RB 101 (20 mg/kg) antinociception. Two-way ANOVA showed a significant treatment effect ($F(3,28) = 132.66$, $P < 0.0001$), time effect ($F(2,56) = 192.69$, $P < 0.0001$), and interaction between treatment and time ($F(6,56) = 59.64$, $P < 0.0001$). One-way ANOVA revealed an effect of the treatment 10 ($F(3,28) = 216.46$, $P < 0.001$), 25 ($F(3,28) = 54.12$, $P < 0.001$), and 40 min ($F(3,28) = 3.76$, $P < 0.05$) after RB 101 administration. Post-hoc comparisons showed that PD-134,308 enhanced significantly the antinociceptive response induced 10 min after RB 101 injection (Fig. 2c).

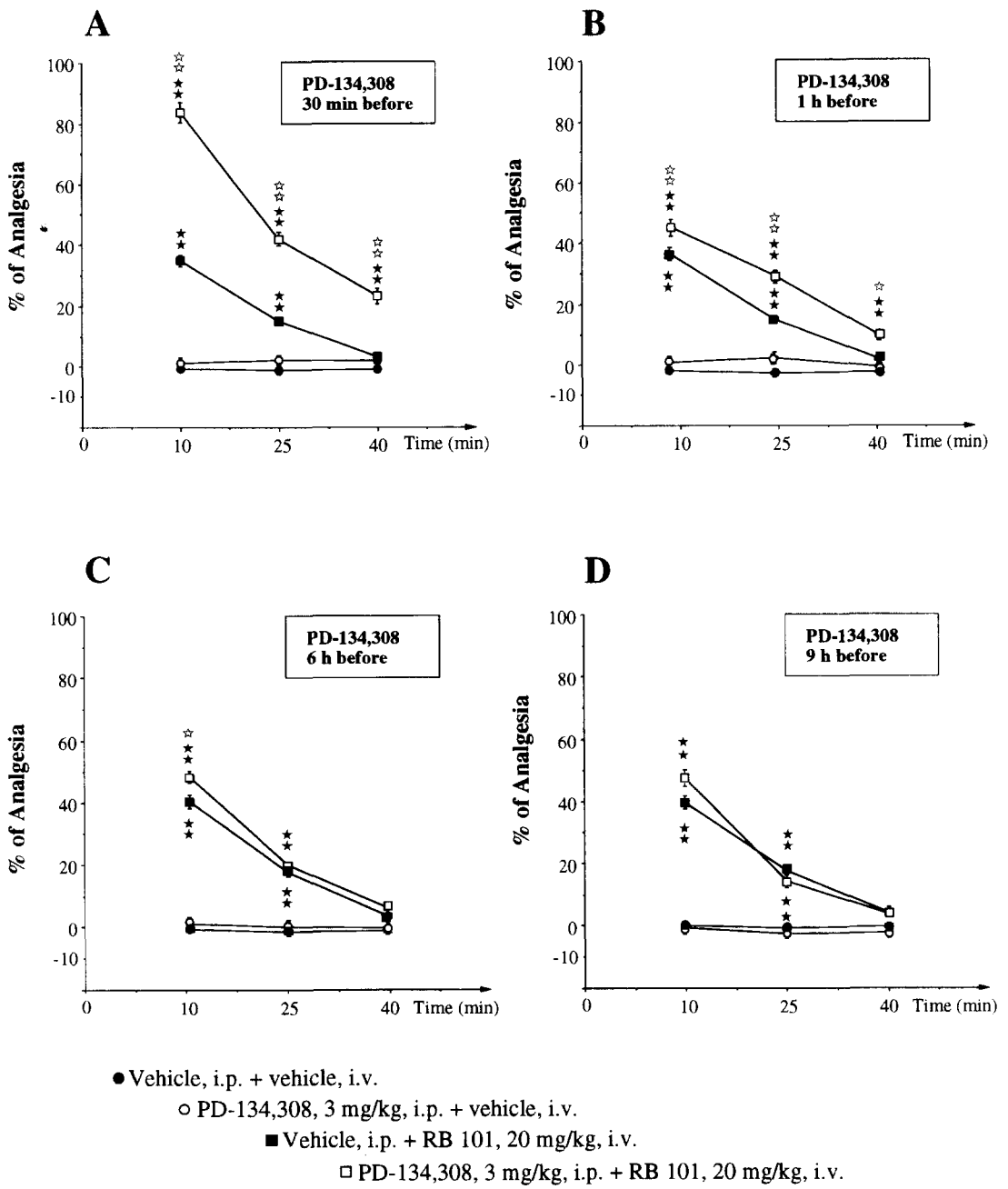
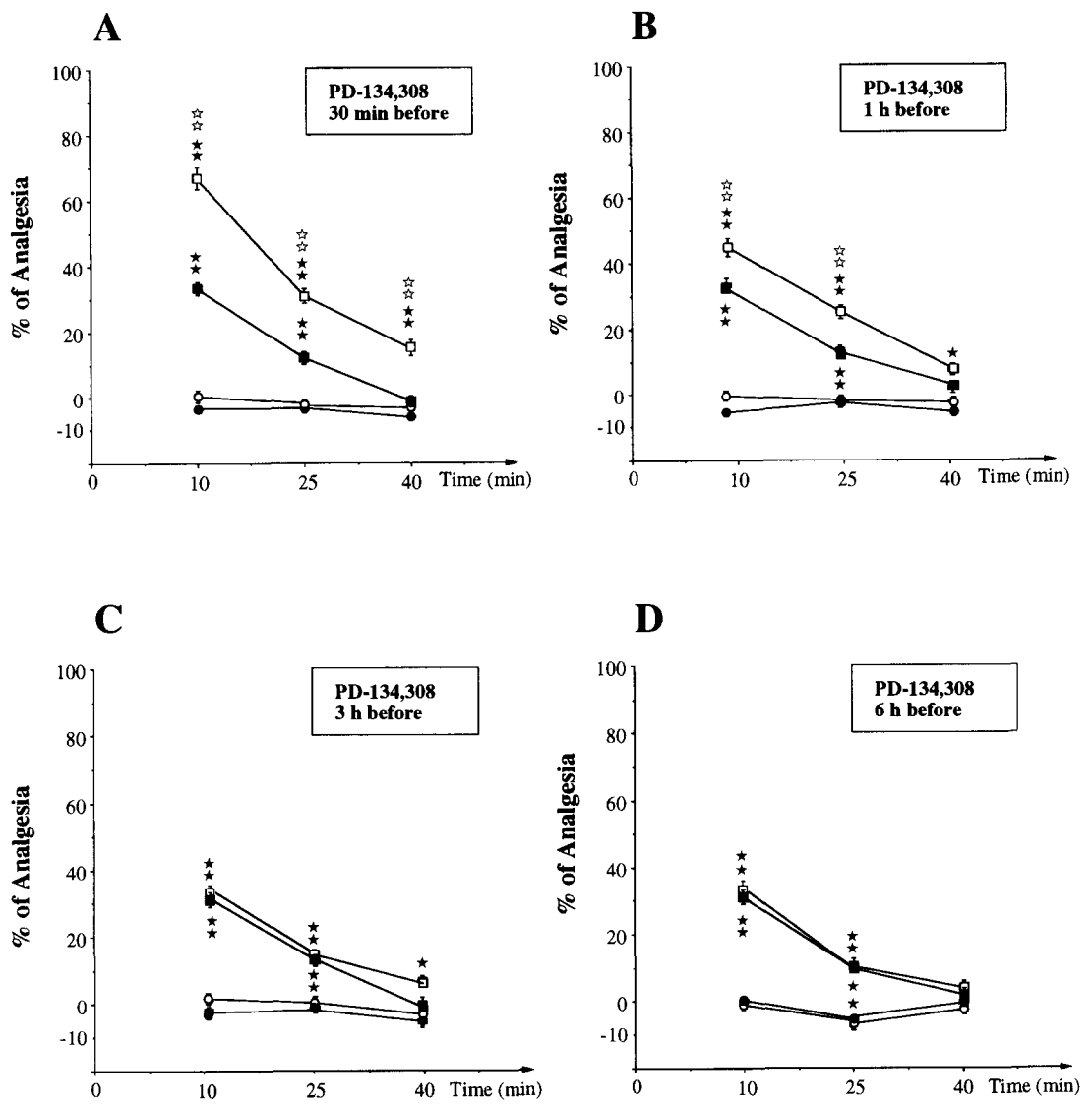


Fig. 2. Effects induced by the pretreatment with PD-134,308 at different time points on the antinociceptive responses induced by RB 101 in the rat tail-flick. PD-134,308 (3 mg/kg, i.p.) was administered 30 min (a), 1 h (b), 6 h (c) or 9 h (d) before the first tail-flick determination. RB 101 (20 mg/kg, i.v.) was injected 10 min before the first tail-flick measurement. Tail-flick latencies were tested at three different times: 10, 25, and 40 min after RB 101 injection. The results are expressed as percentages of analgesia ± S.E.M. ($n = 8$ for each group). ⋆ $P < 0.05$; ⋆⋆ $P < 0.01$ vs. vehicle group, ☆ $P < 0.05$; ☆☆ $P < 0.01$ vs. RB 101 (20 mg/kg) group (Scheffé's F-test).

PD-134,308 (3 mg/kg) administered 9 h before first tail-flick determination did not significantly modify antinociceptive response induced by RB 101 (20 mg/kg). Two-way ANOVA revealed a significant treatment effect ($F(3,28) = 48.09$, $P < 0.001$), time effect ($F(2,56) = 274.52$, $P < 0.001$), and interaction between treatment and time ($F(6,56) = 81.26$, $P < 0.001$). One-way analysis of variance revealed an effect of the treatment 10 ($F(3,28) = 140.29$, $P < 0.001$) and 25 min ($F(3,28) = 27.15$, $P < 0.001$) after RB 101 administration. Individual means comparisons showed that the treatment effects observed at these two times correspond to the



● Vehicle, i.p. + vehicle, i.v.
○ PD-134,308, 1 mg/kg, i.p. + vehicle, i.v.
■ Vehicle, i.p. + RB 101, 20 mg/kg, i.v.
□ PD-134,308, 1 mg/kg, i.p. + RB 101, 20 mg/kg, i.v.

Fig. 3. Effects induced by the pretreatment with PD-134,308 at different time points on the antinociceptive responses induced by RB 101 in the rat tail-flick. PD-134,308 (1 mg/kg, i.p.) was administered 30 min (a), 1 h (b), 3 h (c) or 6 h (d) before the first tail-flick determination. RB 101 (20 mg/kg, i.v.) was injected 10 min before the first tail-flick measurement. Tail-flick latencies were tested at three different times: 10, 25, and 40 min after RB 101 injection. The results are expressed as percentages of analgesia ± S.E.M. ($n = 8$ for each group). ⋆ $P < 0.05$; ⋆⋆ $P < 0.01$ vs. vehicle group, ☆☆ $P < 0.01$ vs. RB 101 (20 mg/kg) group (Scheffé's F-test).

responses induced by RB 101. However, PD-134,308 was not able to significantly enhance this antinociceptive response (Fig. 2d).

3.3. Pharmacokinetic study during 6 h (third experiment)

Tail-flick test in rats

PD-134,308 (1 mg/kg) administered 30 min before first tail-flick determination significantly enhanced RB 101 (20 mg/kg) antinociceptive response. Two-way ANOVA revealed a significant treatment effect ($F(3,24) = 119.20$, $P < 0.001$), time effect ($F(2,48) = 228.55$, $P < 0.001$), and interaction between treatment and time ($F(6,48) = 71.41$, $P < 0.001$). One-way ANOVA showed an effect of the treatment 10 ($F(3,24) = 256.68$, $P < 0.001$), 25 ($F(3,24) = 43.09$, $P < 0.001$), and 40 min ($F(3,24) = 27.27$, $P < 0.001$) after RB 101 administration. Individual means comparisons indicated that PD-134,308 significantly potentiated the antinociceptive effect induced 10, 25 and 40 min after RB 101 injection (Fig. 3a).

PD-134,308 (1 mg/kg) administered 1 h before first tail-flick determination significantly enhanced RB 101 (20 mg/kg) antinociception. Two-way ANOVA revealed a significant treatment effect ($F(3,24) = 54.40$, $P < 0.001$), time effect ($F(2,48) = 153.51$, $P < 0.001$), and interaction between treatment and time ($F(6,48) = 43.16$, $P < 0.001$). One-way ANOVA revealed an effect of the treatment 10 ($F(3,24) = 166.25$, $P < 0.001$), 25 ($F(3,24) = 34.12$, $P < 0.001$), and 40 min ($F(3,24) = 5.19$, $P < 0.001$) after RB 101. Subsequent individual means comparisons indicated that the responses induced 10 and 25 min after RB 101 administration were potentiated by PD-134,308 (Fig. 3b).

PD-134,308 (1 mg/kg) administered 3 h before first tail-flick determination did not significantly modify antinociceptive response induced by RB 101 (20 mg/kg). Two-way ANOVA showed a significant effect of treatment ($F(3,24) = 41.52$, $P < 0.001$), time ($F(2,48) = 169.85$, $P < 0.001$), and interaction between treatment and time ($F(6,48) = 49.20$, $P < 0.001$). One-way ANOVA revealed an effect of the treatment 10 ($F(3,24) = 89.49$, $P < 0.001$), 25 ($F(3,24) = 22.25$, $P < 0.001$), and 40 min ($F(3,24) = 4.31$, $P < 0.05$) after RB 101 administration. Post-hoc comparisons showed that PD-134,308 did not enhance significantly the antinociceptive response induced by RB 101 injection. Indeed, the treatment effects observed at these three times correspond to the responses induced by RB 101 (Fig. 3c).

PD-134,308 (1 mg/kg) administered 6 h before first tail-flick determination did not significantly modify antinociceptive response induced by RB 101 (20 mg/kg). Two-way ANOVA revealed a significant treatment effect ($F(3,24) = 29.85$, $P < 0.001$), time effect ($F(2,48) = 98.13$, $P < 0.001$), and interaction between treatment and time ($F(6,48) = 26.84$, $P < 0.001$). One-way analysis of variance revealed an effect of the treatment 10 ($F(3,24) = 41.90$, $P < 0.001$) and 25 min ($F(3,24) = 21.33$, $P < 0.001$) after RB 101 administration. Individual means comparisons showed that the treatment effects observed at these two times correspond to the responses induced by RB 101. However, PD-134,308 was not able to significantly enhance this antinociceptive response (Fig. 3d).

Hot-plate test in mice

The ability of PD-134,308 (3 mg/kg, i.p.) to enhance the antinociceptive response induced by RB 101 (20

Table 1
Effects induced on RB 101 (20 mg/kg, i.v.) antinociceptive responses by pretreatment with PD-134,308 (3 mg/kg, i.p.) at different times in the hot-plate test in mice

Treatment	% of analgesia mean ± S.E.M.	$P <$
Vehicle + vehicle	0.3 ± 1.9	-
RB 101, 20 mg/kg + vehicle	45.6 ± 4.7	0.01 vs. vehicle
Vehicle + PD-134,308, 3 mg/kg	7.3 ± 2.1	N.S.
RB 101 20 mg/kg + PD-134,308, 3 mg/kg:		
PD-134,308 injected 30 min before test	85.3 ± 3.5	0.01 vs. vehicle 0.01 vs. RB 101
PD-134,308 injected 1 h before test	83.4 ± 4.5	0.01 vs. vehicle 0.01 vs. RB 101
PD-134,308 injected 3 h before test	69.8 ± 6.4	0.01 vs. vehicle 0.05 vs. RB 101
PD-134,308 injected 6 h before test	48.2 ± 4.8	0.01 vs. vehicle N.S. vs. RB 101

Post-hoc individual dose effects were analyzed using Scheffé's F-test.

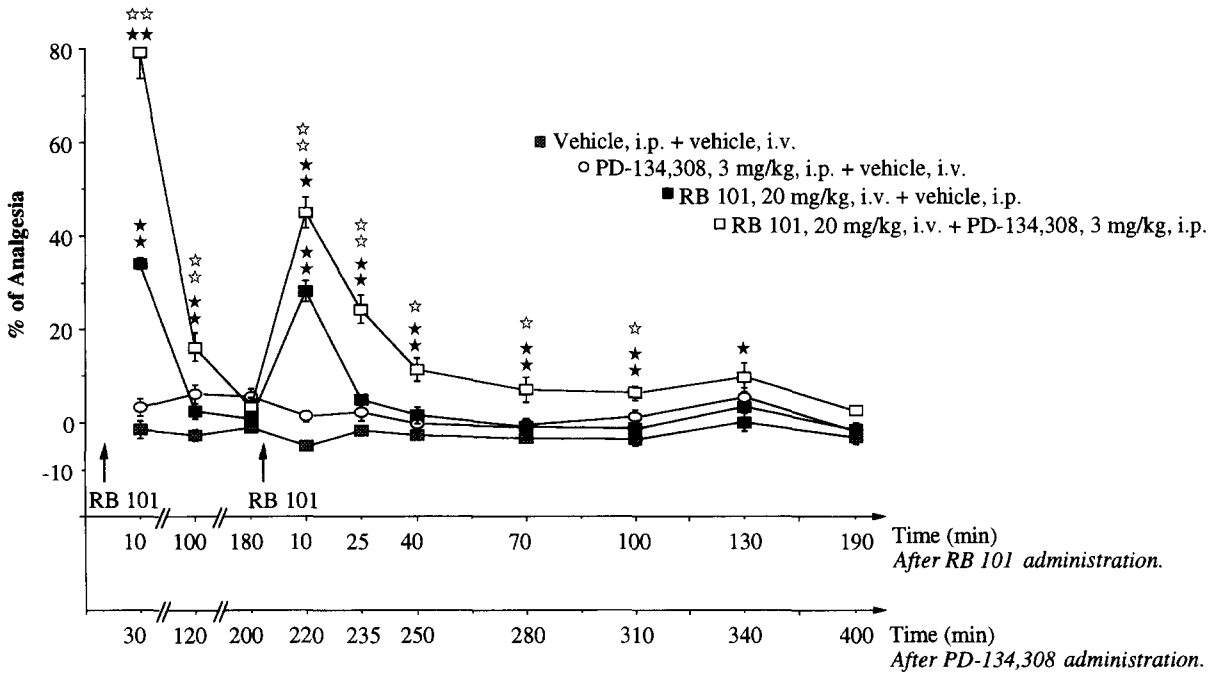


Fig. 4. Effects induced by the CCK_B receptor antagonist PD-134,308 on the antinociceptive responses induced by two repeated administration of RB 101. PD-134,308 (3 mg/kg, i.p.) was administered 30 min before the first tail-flick determination. RB 101 (20 mg/kg × 2, i.v.) was injected 10 min before and 180 min after the first tail-flick measurement. Tail-flick latencies were tested at ten different times: 10, 100 and 180 after the first RB 101 injection, and 10, 25, 40, 70, 100, 130, and 190 min after the second RB 101 injection. The results are expressed as percentages of analgesia ± S.E.M. ($n = 8$ for each group). ⋆ $P < 0.05$; ⋆⋆ $P < 0.01$ vs. vehicle group, ☆ $P < 0.05$; ☆☆ $P < 0.01$ vs. RB 101 (20 mg/kg) group (Scheffé's F-test).

Table 2
Effects induced by the chronic treatment with RB 101 (20 mg/kg), and PD-134,308 (3 mg/kg)

Two-way ANOVA			One-way ANOVA		
Treatment $F(3,35)$	Day $F(2,70)$	Interaction $F(6,70)$	Treatment $F(3,35)$		Day [a]
Time 10 min					
294.39	1.15	0.41	Day 1	90.64	RB 101 + vehicle
$P < 0.001$	N.S.	N.S.		$P < 0.001$	$F(2,27) = 1.29$
			Day 3	90.00	N.S.
				$P < 0.001$	RB 101 + PD-134,308
			Day 5	159.45	$F(2,33) = 0.23$
				$P < 0.001$	N.S.
Time 25 min					
54.82	0.84	2.04	Day 1	46.22	RB 101 + vehicle
$P < 0.001$	N.S.	N.S.		$P < 0.001$	$F(2,27) = 5.97$
			Day 3	18.97	$P < 0.01$
				$P < 0.001$	RB 101 + PD-134,308
			Day 5	37.55	$F(2,33) = 0.57$
				$P < 0.001$	N.S.
Time 40 min					
27.03	1.99	2.82	Day 1	40.46	RB 101 + vehicle
$P < 0.001$	N.S.	$P < 0.05$		$P < 0.001$	$F(2,27) = 4.39$
			Day 3	7.96	$P < 0.05$
				$P < 0.001$	RB 101 + PD-134,308
			Day 5	5.54	$F(2,33) = 5.42$
				$P < 0.01$	$P < 0.01$

Two-way ANOVA with treatment (between subjects) and day (within subjects) as factors of variation. Subsequents one-way ANOVA for treatment (between subjects) and day (within subjects) ($n = 8$–12 animals per group). ANOVA values are given for the three determinations performed in each day of test (10, 25 and 40 min after RB 101 injection). See Methods for details. [a] Vehicle + vehicle and PD-134,308 + vehicle groups did not show significant differences within days.

mg/kg, i.v.) was studied by administering the CCK_B receptor antagonist at different time points: 30 min, 1 h, 3 h and 6 h before the test. One-way ANOVA indicated a significative treatment effect ($F(6,63) = 64.565$, $P < 0.0001$). Post-hoc comparisons showed that the antinociceptive response induced by RB 101 was enhanced by PD-134,308 (3 mg/kg, i.p.) injected 30 min, 1 h and 3 h, but not 6 h, before the hot-plate determination (Table 1).

3.4. Effect induced by a repeated administration of RB 101 on animal pretreated with PD-134,308: pharmacokinetic study during 6 h (fourth experiment)

Two-way analysis of variance revealed a significant treatment effect ($F(3,28) = 50.84$, $P < 0.0001$), time effect ($F(9,252) = 67.66$, $P < 0.0001$), and interaction between treatment and time ($F(27,252) = 25.65$, $P < 0.0001$). The effect induced by PD-134,308 (3 mg/kg) was long enough to potentiate the antinociceptive responses of RB 101 (20 mg/kg) administered 20 or 220 min later. Thus, one-way ANOVA showed an effect of the treatment 10 ($F(3,28) = 137.29$, $P < 0.001$), 100 ($F(3,28) = 16.01$, $P < 0.001$), and 180 min ($F(3,28) = 3.09$, $P < 0.05$) after the first RB 101 administration. The effect of the treatment was also significant 10 ($F(3,28) = 115.51$, $P < 0.001$), 25 ($F(3,28) = 36.70$, $P < 0.001$), 40 ($F(3,28) = 10.22$, $P < 0.001$), 70 ($F(3,28) = 6.36$, $P < 0.01$), 100 ($F(3,28) = 7.94$, $P < 0.001$), 130 ($F(3,28) = 3.27$, $P < 0.05$), and 190 min ($F(3,28) = 3.30$, $P < 0.05$) after the second RB 101 injection. Post-hoc comparisons indicated that PD-134,308 potentiated the antinociceptive effect induced 10 and 100 min after the first administration of RB 101, and 10, 25, 40, 70, and 100 min after the second injection of RB 101 (Fig. 4).

3.5. Antinociceptive effects and possible development of tolerance after chronic administration of RB 101 and PD-134,308 once daily (fifth experiment)

Three-way ANOVA revealed significant treatment ($F(3,35) = 193.44$, $P < 0.0001$) and time effects

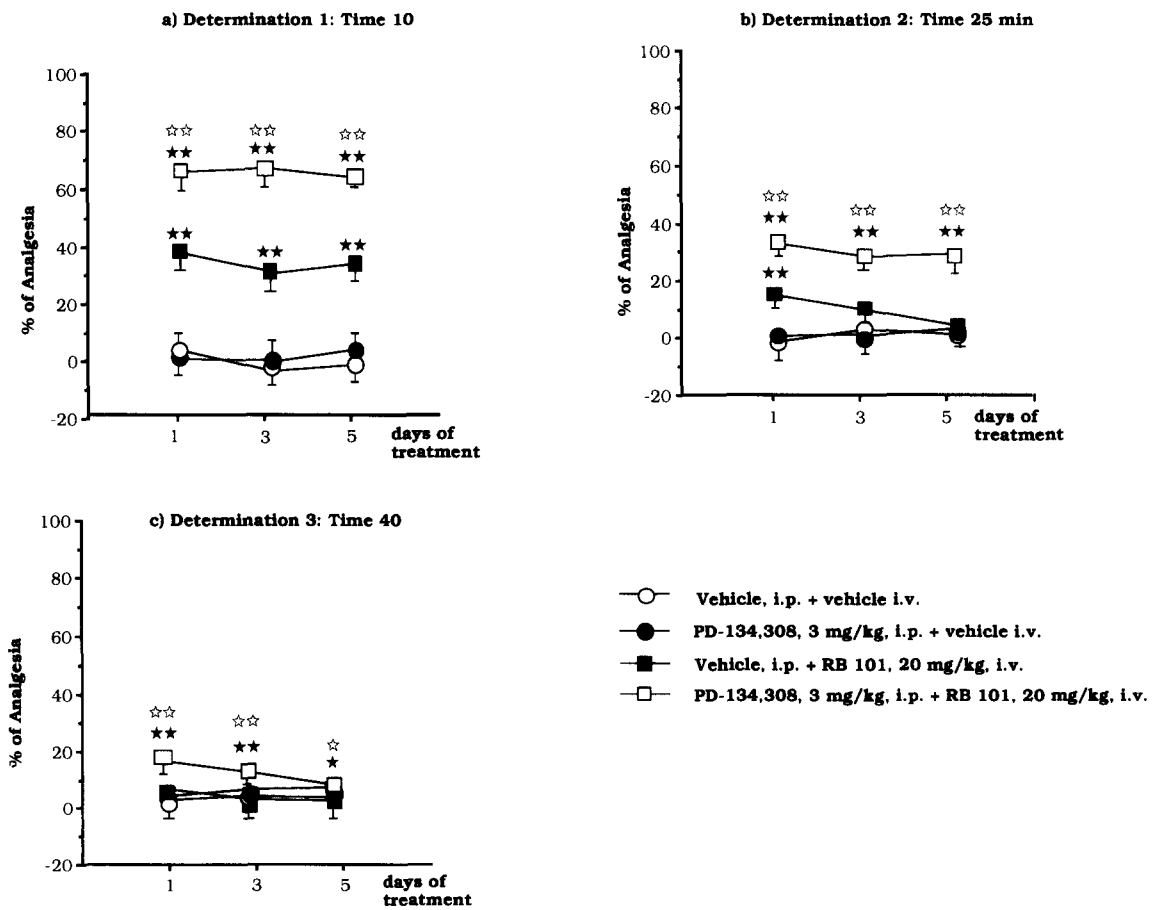


Fig. 5. Effects induced by the chronic treatment during 5 days with the association of RB 101 (20 mg/kg per day, i.v.) and PD-134,308 (3 mg/kg per day, i.p.). Rat tail-flick test was performed on 1st, 3rd and 5th days of treatment. Three determinations were performed in each day of test: 10 (determination 1) (a), 25 (determination 2) (b) and 40 (determination 3) (c) min after RB 101 injection. The results are expressed as percentages of analgesia ± S.E.M. ($n = 8$–12 for each group). ★ $P < 0.05$; ★★ $P < 0.01$ vs. vehicle group, ☆ $P < 0.05$; ☆☆ $P < 0.01$ vs. RB 101 (20 mg/kg) group (Scheffé's F-test).

($F(2,70) = 76.05$, $P < 0.0001$), without day effect ($F(2,70) = 0.24$, N.S.). Interactions treatment/time ($F(6,70) = 38.97$, $P < 0.0001$), day/time ($F(4,140) = 132.91$, $P < 0.0001$) and treatment/time/day ($F(12,140) = 56.63$, $P < 0.0001$) were significant, but not interaction treatment/day was observed ($F(6,70) = 0.78$, N.S.). Two-way ANOVA (treatment and day) and subsequent one-way ANOVA are shown in Table 2. The evolution of the three latency measures (10, 25 and 40 min after RB 101 injection) revealed that the maximal antinociceptive effect induced by RB 101 (10 min after injection) given alone or coadministrated with PD-134,308 was not modified during the 5 days of treatment. However, post-hoc comparisons indicated a diminution in the duration of the response. Thus, in the second determination (25 min after RB 101 injection) RB 101 exhibited a significant antinociceptive effect on day 1, which decreased progressively on days 3 and 5, when it represented the 32% of the initial response (day effect: 1st vs. 3rd day, N.S.; 1st vs. 5th day, $P < 0.01$). The antinociception induced by the association of RB 101 plus PD-134,308 was similar during the 5 days of treatment in the second determination. In the third determination (40 min after RB 101), the antinociceptive effect of RB 101 was not significant on any day of treatment. In this determination, the coadministration of RB 101 and PD-134,308 exhibited an antinociceptive effect on the 1st day ($P < 0.01$) which was slightly decreased on the 3rd day, and significantly diminished on the 5th day (1st vs. 5th day, $P < 0.01$). However, the antinociception remained significant on day 5, representing 39% of the initial value (Fig. 5).

3.6. Antinociceptive effects and possible development of tolerance after chronic administration of RB 101 and PD-134,308 twice daily (sixth experiment)

Three-way ANOVA revealed significant treatment ($F(3,32) = 284.37$, $P < 0.0001$), time ($F(2,64) = 64.31$, $P < 0.0001$) and day effects ($F(2,64) = 84.74$, $P < 0.0001$). Interactions treatment/time ($F(6,64) = 20.05$, $P < 0.0001$), treatment/day ($F(6,64) = 35.41$, $P < 0.0001$), day/time ($F(4,128) = 225.97$, $P < 0.0001$) and treatment/time/day ($F(12,128) = 112.94$, $P < 0.0001$) were significant. Two-way ANOVA (treatment and day) and subsequent one-way ANOVA are shown in Table 3. The evolution of the three latency measures (10, 25 and 40 min after RB 101 injection) revealed that the maximal antinociceptive effect induced by RB 101 (10 min after injection) given alone or coadministrated with PD-134,308 was not modified during the 5 days

Table 3
Effects induced by the chronic treatment with RB 101 (20 mg/kg, twice daily), and PD-134,308 (3 mg/kg, twice daily)

Two-way ANOVA			One-way ANOVA		
Treatment $F(3,32)$	Day $F(2,64)$	Interaction $F(6,64)$	Treatment $F(3,22)$		Day [a]
Time 10 min					
232.92	8.56	3.32	Day 1	90.64	RB 101 + vehicle
$P < 0.001$	$P < 0.001$	$P < 0.001$		$P < 0.001$	$F(2,24) = 3.04$
			Day 3	90.00	N.S.
				$P < 0.001$	RB 101 + PD-134,308
			Day 5	159.45	$F(2,24) = 3.13$
				$P < 0.001$	N.S.
Time 25 min					
73.77	16.73	1.83	Day 1	46.22	RB 101 + vehicle
$P < 0.001$	$P < 0.001$	N.S.		$P < 0.001$	$F(2,24) = 6.67$
			Day 3	37.42	$P < 0.01$
				$P < 0.001$	RB 101 + PD-134,308
			Day 5	17.69	$F(2,24) = 4.12$
				$P < 0.01$	$P < 0.05$
Time 40 min					
48.14	9.87	4.15	Day 1	34.62	RB 101 + vehicle
$P < 0.001$	$P < 0.001$	$P < 0.01$		$P < 0.001$	$F(2,24) = 0.62$
			Day 3	27.53	N.S.
				$P < 0.001$	RB 101 + PD-134,308
			Day 5	5.58	$F(2,24) = 8.40$
				$P < 0.01$	$P < 0.01$

Two-way ANOVA with treatment (between subjects) and day (within subjects) as factors of variation. Subsequents one-way ANOVA for treatment (between subjects) and day (within subjects) ($n = 8$–10 animals per group). ANOVA values are given for the three determinations performed in each day of test (10, 25 and 40 min after RB 101 injection). See Methods for details. [a] Vehicle + vehicle and PD-134,308 + vehicle groups did not show significant differences within days.

with this pause of treatment. However, post-hoc comparisons indicated a decrease in the duration of the RB 101 response similar to that observed on experiment 5. The effect in the second determination (25 min after RB 101 injection) on day 5 represented the 25% of the initial response (day effect: 1st vs. 3rd day, N.S.; 1st vs. 5th day, $P < 0.01$). The antinociceptive effect induced by the association of RB 101 plus PD-134,308 also was decreased on day 5, when it represents the 59% of the initial response (1st vs. 3rd day, N.S.; 1st vs. 5th day, $P < 0.05$). In the third determination (40 min after RB 101), the antinociceptive response of RB 101 was not significant on any day of treatment. In this determination, the coadministration of RB 101 and PD-134,308 exhibited an antinociceptive effect on the 1st day ($P < 0.01$) which was significantly decreased on the 5th day (1st vs. 5th day, $P < 0.01$). The response was not significant on day 5, representing 36% of the initial value (Fig. 6).

4. Discussion

We reported previously that different selective CCK_B receptor antagonists (PD-134,308, L-365,260 and RB 211) strongly potentiate the antinociceptive responses induced by RB 101, a full inhibitor of enkephalin-degrading enzymes able to cross the blood-brain barrier (Fournié-Zaluski et al., 1992), in both tail-flick and hot-plate tests (Maldonado et al., 1993; Valverde et al., 1994). In the present study, the duration of this effect and its possible development of tolerance have been investigated. The antinociceptive test used was the rat tail-flick, since this test has been shown to be the most sensitive to the facilitatory effect induced by CCK_B receptor antagonists on RB 101 responses (Valverde et al., 1994). However, the hot-plate test was also used in order to verify the relative importance of spinal and supraspinal structures in the potentiation of RB 101-produced antinociception by

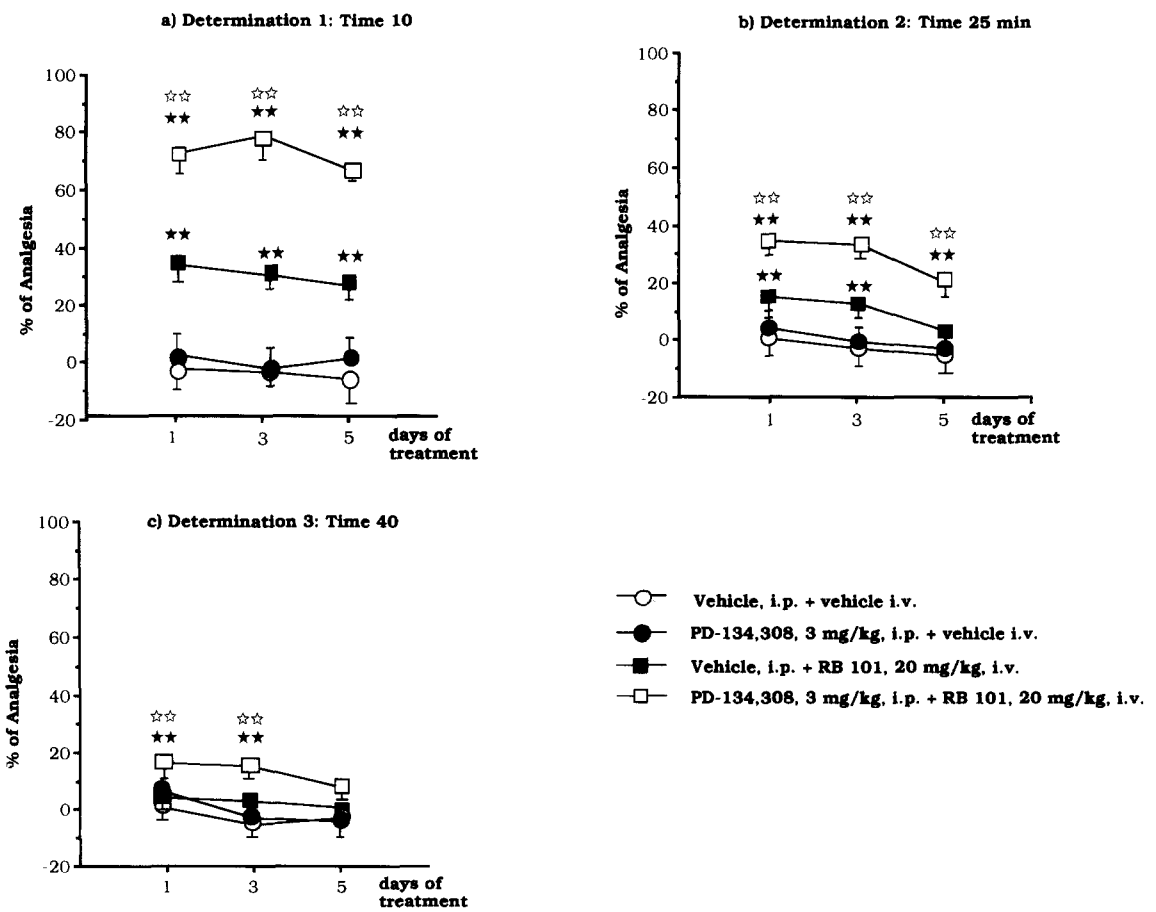


Fig. 6. Effects induced by the chronic treatment during 5 days with the association of RB 101 (20 mg/kg twice daily, i.v.) and PD-134,308 (3 mg/kg twice daily, i.p.). Rat tail-flick test was performed on 1st, 3rd and 5th days of treatment. Three determinations were performed in each day of test: 10 (determination 1) (a), 25 (determination 2) (b) and 40 (determination 3) (c) min after RB 101 injection. The results are expressed as percentages of analgesia ± S.E.M. ($n = 8$–10 for each group). ★ $P < 0.05$; ★★ $P < 0.01$ vs. vehicle group, ☆ $P < 0.05$; ☆☆ $P < 0.01$ vs. RB 101 (20 mg/kg) group (Scheffé's F-test).

CCK_B receptor antagonists. A elevate dose of RB 101 (20 mg/kg) was chosen in this study in order to investigate the maximal response in term of duration of action. The CCK_B receptor antagonist selected for this study was PD-134,308 because it induces the strongest effect, and unlike other antagonists, this effect is dose-dependent (Valverde et al., 1994). PD-134,308 prolonged (130 min) 5-fold, the duration of the analgesia produced by a single systemic injection of RB 101 in the tail-flick test. The peak effect of this potentiation was observed at 30 min after PD-134.308 administration. In the case of L-365,260, the potentiation observed was weaker and disappeared after 70 min.

The enhancement of endogenous enkephalin antinociception was not due to an intrinsic antinociceptive effect of CCK_B receptor blockade, as PD-134,308 or L-365,260 have no effect on the pain threshold when given alone. Previous studies reported a weak naloxone sensitive antinociceptive effect induced by PD-134,308 administration (1 mg/kg, i.p.) (Wiesenfeld-Hallin et al., 1990), but this response was not found in a posterior study (Hoffmann and Wiesenfeld-Hallin, 1994).

Until now, no studies have been performed to investigate in detail the duration of the responses obtained by the association of CCK receptor antagonists and exogenous and endogenous opioids on the control of nociception. Zhou et al. (1993) showed an increase in the time course of morphine (4 mg/kg, s.c.)-or ohmefentanyl (32 ng, i.t.)-induced antinociception after devazepide or L-365,260 administration during a period of 60 min. A similar duration of the effects induced by L-365,260 on endogenous enkephalin-induced antinociception was observed in the present study. The administration of a high, and probably non-specific dose of the CCK_A receptor antagonist devazepide prolonged the duration of the morphine (4 mg/kg, i.p.) effect only during a period of about 40 min (Dourish et al., 1988), whereas Lavigne et al. (1992) reported that L-365,260 or high doses of devazepide prolonged morphine (3.5 mg/kg, i.p.) effects for about 120 min. Moreover, Xu et al. (1992) have recently described that PD-134,308 (1 mg/kg) prolonged for about 180 min the depression of the flexor reflex induced by a i.v. injection of morphine in rats with intact sciatic nerves.

In the hot-plate test, PD-134,308 potentiated RB 101 antinociceptive effects when injected 3 h but not 6 h before the test, and the enhancement induced was smaller than that observed in the tail-flick. Consequently, the facilitatory effects were observed at both spinal and supraspinal levels, but the spinal participation seems to be greater, in agreement with the possible better penetration of PD-134,308 into the spinal cord. The methodology used could also participate in the different effects observed on the hot-plate and the tail-flick tests. Thus, a different group of animals was used on mouse hot-plate test for each time-point in order to avoid the well-known development of tolerance to the test. However, in the rat tail-flick paradigm, a within subjects design (testing several times the same animal) was used since no tolerance to the test is developed under these conditions. The repeated exposure to the tail-flick test may induce an habituation of the animals. This habituation may produce changes in the opioid and/or endogenous CCK systems, leading to a facilitation of the antinociceptive response induced by the association of RB 101 and PD-134,308. This may be particularly important in our case, considering the anxiolytic characteristics of RB 101 (Baamonde et al., 1992) and PD-134,308 (Hughes et al., 1990; Derrien et al., 1994b). In agreement with this, an antinociceptive response was induced by PD-134,308 on rats habituated to the hot-plate (Wiesenfeld-Hallin et al., 1990) whereas no intrisic effect was found in the present study on mice hot-plate.

Previous studies reported that CCK receptor antagonists attenuate the development of tolerance to antinociception induced by exogenous opiate administration (Watkins et al., 1984; Faris et al., 1986; Dourish et al., 1990; Xu et al., 1992; Ding and Bayer, 1993; Zhou et al., 1993; Rezayat et al., 1994). This effect seems to be selectively mediated through CCK_B receptors in rodents (Dourish et al., 1990; Rezayat et al., 1994). However, the involvement of spinal CCK_A receptor cannot be excluded in primates (Xu et al., 1992). In our study, PD-134,308 injected only once was able to potentiate during a period of at least 5 h the antinociceptive responses induced by two repeated administration of RB 101. The duration and intensity of the antinociception induced by the first and second RB 101 injection was very similar. This result suggests that in this time period no tolerance occurred to this facilitatory response. The development of tolerance was also investigated under chronic conditions. Thus, RB 101 given alone, once or twice daily during 5 days induced an antinociceptive response which peak effect remained unchanged during the whole period in both shedules of treatment. This result is in agreement with a previous study showing that repeated i.p. administration of RB 101, does not induce tolerance to maximal antinociceptive effect in hot-plate test in mice (Noble et al., 1992b). However, a decrease in the duration of the antinociceptive response of RB 101 was observed in this study during the chronic treatment, reflecting a partial development of tolerance. When RB 101 and PD-134,308 were chronically administered, a similar result was observed. Indeed, the peak effect was not modified during the chronic treatment but a decrease in the duration of the response was observed. However this decrease was smaller than that reported with RB 101 alone and was more intense when the association of RB 101 and PD-134,308 was injected twice daily than when only one administration was given. Indeed,

when the association of both compounds was given once a day, the decrease in the antinociceptive response was only produced at the third determination (40 min after RB 101 injection), whereas when the association was administered twice a day, the diminution in the effects was observed at both, the second (25 min after RB 101 injection) and the third (40 min after RB 101 injection) determinations.

The molecular mechanisms for the prevention of opioid tolerance by CCK remain unknown. Several authors (Faris et al., 1983; Galina and Kastin, 1986; Malin et al., 1990) consider an antiopioid model of tolerance. This model suggests that after morphine administration, the brain synthesizes and secretes neuropeptides as CCK, which decrease the pharmacological effects of morphine. This release of CCK may constitute a self-limiting process for opioid effects (Noble et al., 1993; Zhou et al., 1993). Consequently, removal of the effect of CCK would result in an augmentation of opioid analgesia and reversal of opiate tolerance. This theory could explain the attenuation of RB 101 tolerance induced by the CCK_B receptor antagonists. Indeed, PD-134,308 may minimize the mechanisms involved in the decrease of the duration of RB 101 antinociception, that could implicate changes on the enkephalins release, on the opioid receptors and/or on the post-receptor transduction cascade.

It has been also suggested that tolerance to the analgesic effect of opiates may be related to classical conditioning (Siegel and McRae, 1984; Dafters and Odber, 1989). Tolerance is interpreted as a manifestation of the acquisition of a compensatory response between the pharmacological effect of the drug and environmental cues (drug administration) which always precede these pharmacological effects. In line with this theory, Xu et al. (1992) have hypothetized that since CCK_B receptor antagonists are anxiolytic agents (Derrien et al., 1994a), these drugs might depress the reaction and memory of animals to negative environmental cues, resulting in the prevention of tolerance. In contrast with this theory, after the administration of RB 101 and PD-134,308, we observed a tolerance only in the third tail-flick determination performed 40 min after RB 101 injection, but not in the first and second determinations, measured 10 and 25 min after the injection. The classical conditioning hypothesis cannot explain this effect of the CCK_B receptor antagonist since if the memory of animals to the drug administration has been abolished 10 and 25 min after the injection it is difficult to conceive that this memory could be re-established 40 min later.

The present findings provide interesting clinical perspectives because different parallel actions have been observed: first, the strong increase induced by PD-134,308 on the peak antinociceptive effect of RB 101 (Valverde et al., 1994). Second, the long-lasting duration of the facilitatory effect observed with this association, prolonging 5-fold the duration of RB 101 response given alone and finally, the peak of the antinociceptive effects induced by the association of RB 101 and PD-134,308 did not change during chronic treatment. The therapeutic perspectives of this association are also supported by the few opiate drawbacks reported following chronic RB 101 administration (Noble et al., 1992a; review in Roques et al., 1993). Furthermore, CCK_B receptor antagonists (Hughes et al., 1990; Daugé et al., 1993; Harro et al., 1993; Derrien et al., 1994a; Hernando et al., 1994) as well as RB 101 (Baamonde et al., 1992) have been shown to elicit anxiolytic properties and to be effective in some animal models of depression (Baamonde et al., 1992; Derrien et al., 1994b; Hernando et al., 1994). Chronic pain usually produces a high degree of anxiety in man, that in turn may increase pain sensation (Kocher, 1976). Consequently, PD-134,308 associated with RB 101 could improve this emotional component, which seems to be crucial in the maintenance of chronic pain in man. In conclusion, co-administration of RB 101 and CCK_B receptor antagonist may represent a perspective to fill the still uncovered domain between non-opiates and classical opiate analgesics.

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