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Antinociception produced by the peptidase inhibitor, RB 101, in rats with adrenal medullary transplant into the spinal cord

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Abstract

This study was undertaken to investigate the effects induced by the systemic administration of RB 101 [*N*-{(*R*,*S*)-2-benzyl-3[(*S*)(2-amino-4-methylthio)butyl dithio]-1-oxoprpyl}-L-phenylalanine benzyl ester], a mixed inhibitor of the enkephalin catabolism able to cross the blood–brain barrier, in antinociception produced by adrenal medullary tissue transplanted in the rat spinal subarachnoid space. For this purpose, the antinociceptive responses induced by intravenous (i.v.) administration of RB 101 were evaluated in the tail-flick in rats transplanted 28 and 56 days before the test. Systemic administration of RB 101 induced antinociceptive effects in sham-operated rats, as previously reported. RB 101 also enhanced significantly the antinociception produced by the autotransplant 28 and 56 days after surgery. The antinociceptive responses of RB 101 in both sham-operated and autotransplanted rats were blocked by naloxone, but were not modified by the noradrenergic antagonist, phentolamine, suggesting a selective involvement of opioid mechanisms. The present results indicate that the inhibitors of enkephalin catabolism enhance the antinociception induced by adrenal medullary transplants.

Keywords: RB 101; Adrenal medullary transplant; Enkephalin; Antinociception; Tail-flick; Naloxone; Phentolamine

1. Introduction

Several experimental and clinical studies suggest that cell transplantation into the central nervous system may serve as a useful therapeutic approach to supplement neurotransmitter levels in some human nervous system disorders (Freed et al., 1981; Perlow, 1987). Transplants of adrenal medullary chromaffin cells into the spinal cord subarachnoid space are reported to be a sustained source for the spinal release of neurochemical substances that inhibit pain. Long-term pain relief following transplantation is reported in several acute and chronic experimental models of nociception (Sagen et al., 1986, 1990). In particular, chromaffin cells synthesize and secrete opioid peptides, including [Met^5]- and [Leu^5]enkephalin, and catecholamines (Eiden et al., 1984) which independently reduce pain when injected into the spinal subarachnoid space (Reddy et al., 1980). Furthermore, adrenal medullary transplants have been shown to elevate [Met^5]enkephalin (Sagen and Kemmler, 1989) and catecholamine levels (Sagen and Wang, 1990) in cerebrospinal fluid. The basal release of opioid peptides and catecholamines from these transplants is apparently sufficient for the reduction of pain in some experimental models, such as chronic arthritic rats (Sagen et al., 1990) and neuropathic pain (Hama and Sagen, 1993).

Various experimental procedures have been suggested to increase the antinociceptive effects produced by adrenal medullary transplants and/or to prolong the duration of

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the analgesic response. Thus, the level of neuroactive substances released from the transplanted cells can be increased by stimulating nicotinic receptors (Eiden et al., 1984), and a potent antinociception has been revealed in transplanted animals following systemic injections of low doses of nicotine (Sagen et al., 1986). Since the opioid peptides released from the transplants are rapidly degraded, limiting the time course of their effectiveness, another approach to improve this antinociceptive response consisted of administering an inhibitor of enkephalin catabolism directly into the subarachnoid space (Sagen and Wang, 1990). Indeed, the administration of the mixed inhibitor of the enkephalin catabolism, kelatorphan (Fournié-Zaluski et al., 1984), by an intrathecal route prolonged nicotine-stimulated antinociception in rats with adrenal medullary transplants (Sagen and Wang, 1990). Recently, a potent mixed inhibitor of enkephalin catabolism, able to cross the blood-brain barrier, RB 101 [*N*-{(*R*,*S*)-2-benzyl-3[(*S*)(2-amino-4-methylthio)butyl dithio]-1-oxoprpyl}-L-phenylalanine benzyl ester], has been synthesized (Fournié-Zaluski et al., 1992). This compound is a prodrug which generates a potent inhibitor of the aminopeptidase *N*,(*S*)2-amino-1-mercapto-4-methylthio butane, and another of the neutral endopeptidase, *N*-[(*R*,*S*)-2-mercaptomethyl-1-oxo-3-phenylpropyl]-L-phenylalanine, through a biologically dependent cleavage of its disulphide bond. RB 101 is the first mixed inhibitor able to induce strong antinociceptive responses in mice and rats after systemic administration (Fournié-Zaluski et al., 1992; Noble et al., 1992a) by elevating the extracellular level of endogenous enkephalins (Ruiz-Gayo et al., 1992; Daugé et al., 1996). The opportunity to use this compound by a peripheral route is a promising step towards the development of new therapeutic tools.

The present study was undertaken to investigate whether the systemic administration of the mixed inhibitor of the enkephalin catabolism, RB 101, is able to enhance the antinociception induced in the tail-flick test by adrenal medullary tissue transplanted in the rat spinal subarachnoid space. The participation of noradrenergic and opioid systems on the antinociception induced by these experimental procedures has been investigated by using naloxone and phentolamine as antagonists of opioid and noradrenaline receptors, respectively.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 220–240 g at the beginning of the experiment, and supplied by Depré (France) were used in this study. They were housed in groups of five, and maintained in a controlled environment with water and food made available ad libitum. Animals were allowed to adapt to the animal room for at least 1 week prior to use. Animal care complied with that stipulated by local committee and international organisations (IASP, 1983; NIH Publication No. 85-23, 1985).

2.2. Drugs

The peptidase inhibitor, RB 101 (mesylate salt), was synthesized in our laboratory as described (Fournié-Zaluski et al., 1992). Naloxone, phentolamine and cremophor EL were obtained from Sigma laboratories. RB 101 was dissolved in the following vehicle: ethanol (10%), cremophor EL (10%) and distilled water (80%). Naloxone and phentolamine were dissolved in saline (0.9% NaCl). All the compounds were administered in a volume of 1 ml/kg.

2.3. Intravenous administration procedure

Intravenous (i.v.) injections (RB 101 and vehicle) were performed through the dorsal vein of the tail. For this purpose, animals were gently immobilized using a plastic container (Iffa Credo, L'Arbresle, France). The dorsal vein of the tail was dilated by using a cotton impregnated with warm water. In order to avoid stress, animals were habituated to the restraint plastic container before the first tail-flick determination. After this habituation, the restraint procedure had no effect in tail-flick performance since the latencies after saline i.v. administration were similar to those obtained before such an injection (see Figs. 3 and 4).

2.4. Tail-flick procedure

The antinociceptive responses were determined by measuring the time required to respond to 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 automatized tail-flick analgesymeter (Apelex, France) was used. The cut-off time was set at 15 s. For each rat, three determinations were carried out at each time point. The tail-flick latency responses were expressed as a percentage of analgesia calculated by: percent analgesia = (test latency − basal latency)/(cut-off time − basal latency) × 100. The intensity of the thermal stimulus was adjusted to obtain a control latency between 4 and 6 s.

2.5. Autotransplant procedure

The autotransplant was performed as previously described (González-Darder and Ruz-Franzi, 1991; Ruz-Franzi and Gonzalez-Darder, 1991). Briefly, the rats were anaesthetised with chloral hydrate (6 mg/kg, i.p.). The access to the lumbar subarachnoid space was obtained via a dorsal incision and a lumbar laminectomy. The left adrenal gland was dissected out, and the medulla was

subsequently implanted into the subarachnoid lumbar space. The group of sham-operated rats was similarly processed. Thus, sham operations consisted in removing the left adrenal gland, but the adrenal medulla tissue was not implanted into the subarachnoid space. The rats were then left to recover and included in any of the experimental groups stated above. As a control of the welfare of the animals, they were daily observed and periodically weighed. Rats showing any sign of illness were excluded from the study and immediately sacrificed in order to avoid suffering.

2.6. Antinociceptive studies

Before starting the experimental sequence, all the animals were tested on the tail-flick test. They were then either sham-operated or autotransplanted (see protocols above) and caged (three rats per cage). The animals were subjected to subsequent tail-flick tests depending on the experiment. All the animals were evaluated by an observer who did not know either the animal treatment or the surgical procedure performed. A decrease in the tail-flick latencies of control groups (from 5 to 20%) was observed in the subsequent tail-flick determinations when compared to the pre-surgery control. However, this decrease was not significant in any group of animals.

2.6.1. Experiment 1

This experiment was performed in order to determine the effects of RB 101 on antinociception induced after 28 days of adrenal medullary transplant. Twenty-seven rats were sham-operated and 27 were autotransplanted. The animals were then left to recover, and periodically observed and weighted until day 28. To check the effect of the transplant by itself, tail-flick responses were tested in all the rats 10 min before drug administration at day 28. After this tail-flick test, RB 101 was i.v. administered at the doses of 1.66 and 5 mg/kg in both sham-operated and autotransplanted rats. The effects of RB 101 on the tail-flick nociceptive threshold were tested at 10, 20, 30, 40 and 70 min after administration. Proper control groups treated with saline (0.9% NaCl) were processed in order to determine the effects of experimental manipulation. Therefore, the following groups were included in this experiment: sham + saline ($n = 9$), sham + 1.66 mg/kg of RB 101 ($n = 9$), sham + 5 mg/kg of RB 101 ($n = 9$), autotransplanted + saline ($n = 9$), autotransplanted + 1.66 mg/kg of RB 101 ($n = 9$), autotransplanted + 5 mg/kg of RB 101 (n 9).

2.6.2. Experiment 2

The purpose of this experiment was to determine the responses of RB 101 on nociception induced after 56 days of adrenal medullary transplant, and to evaluate the effects of naloxone or phentolamine pretreatment on these responses. Thirty-eight rats were sham-operated and 40 were autotransplanted. On day 56, the animals were tested in the tail-flick 10 min before the administration of RB 101 (5 mg/kg, i.v.), and 10, 20, 30, 40 and 70 min after this injection. Naloxone (1 mg/kg, s.c.) and phentolamine (10 mg/kg, s.c.) were administered 5 min before the administration of the peptidase inhibitor. The following groups were included in this experiment: sham + saline + saline ($n = 11$), sham + RB 101 + saline ($n = 11$), sham + RB 101 + naloxone ($n = 8$), sham + RB 101 + phentolamine ($n = 8$), autotransplanted + saline + saline ($n = 12$), autotransplanted + RB 101 + saline ($n = 12$), autotransplanted + RB 101 + naloxone ($n = 8$), autotransplanted + RB 101 + phentolamine ($n = 8$).

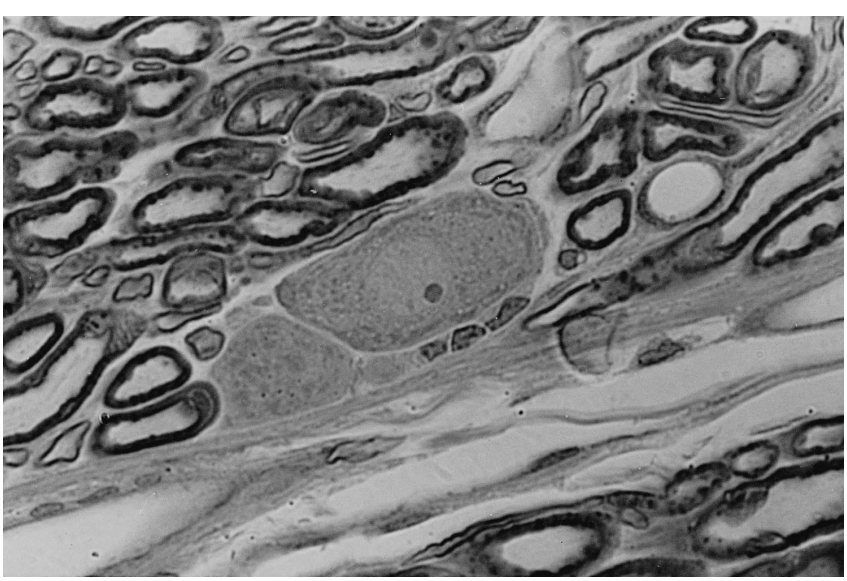

Fig. 1. Histological appearance (optic micrographs) of two grafted cromaffin cells, 2 months after transplantation in the subarachnoid space of the spinal cord. Haemotoxylin and eosin stain. Original magnification × 100.

2.7. Electron microscopy procedure

For the electron microscopy verification, two animals per group were used following completion of the experimental sequence. The animals were deeply anaesthetized with sodium pentobarbital (40 mg/kg), injected with 3 ml of a heparin solution (5000 U.I./ml), and perfused with a solution containing 4% glutaraldehyde, 6% paraformaldehyde and 0.2% $Ca\ Cl_2$ in 0.1 M of phospho buffer solution at pH 7.4. A total volume of 600 ml of this solution was perfused at a rate of 20 ml/min. The spinal cords were removed, and the tissue that presumably contained the adrenal medullary transplant was identified by using a magnifying glass, and isolated. The tissues were fixed with 2% OsO_4 in 0.1 M of phospho buffer solution at pH 7.4 for 1 h at 4°C.

After dehydration in series of ethanol solutions and treatment with propylene oxide, the tissues were included in Epoxy LX 112 resin (Ladd-Janning, Rueil-Malmaison, France). Semithin sections (1 μm) were made with Ultracut (Reichert-Jung) and stained with toluidine blue. Thin sections (60–100 nm) were made in areas containing the cromaffin cells, contrasted with uranyl acetate (2% in ethanol) and lead citrate solution (lead citrate 2%, lead nitrate 1.5% and sodium hydrate 0.5% in distilled water), and observed with a transmission electron microscope (Jeol type JEM 100 S).

2.8. Statistical analysis

The results obtained from the antinociceptive studies are expressed as the mean ± S.E.M. of the percentage of change in pain threshold as compared to the sham-operated treated group (time—10 min) of the same experiment. For

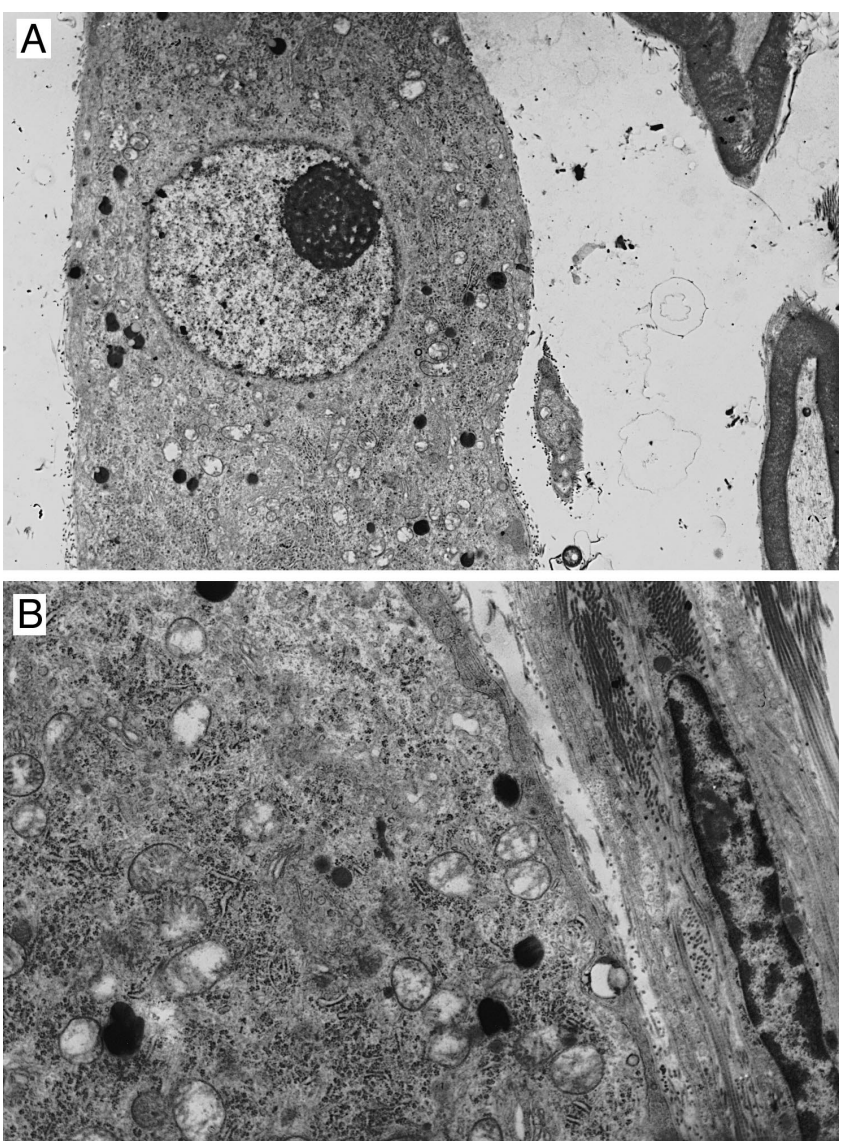

Fig. 2. Electron micrographs of section showing grafted cromaffin cells, 2 months after transplantation in the subarachnoid space of the spinal cord. Original magnification: A × 3000; B × 6000; C × 15 000.

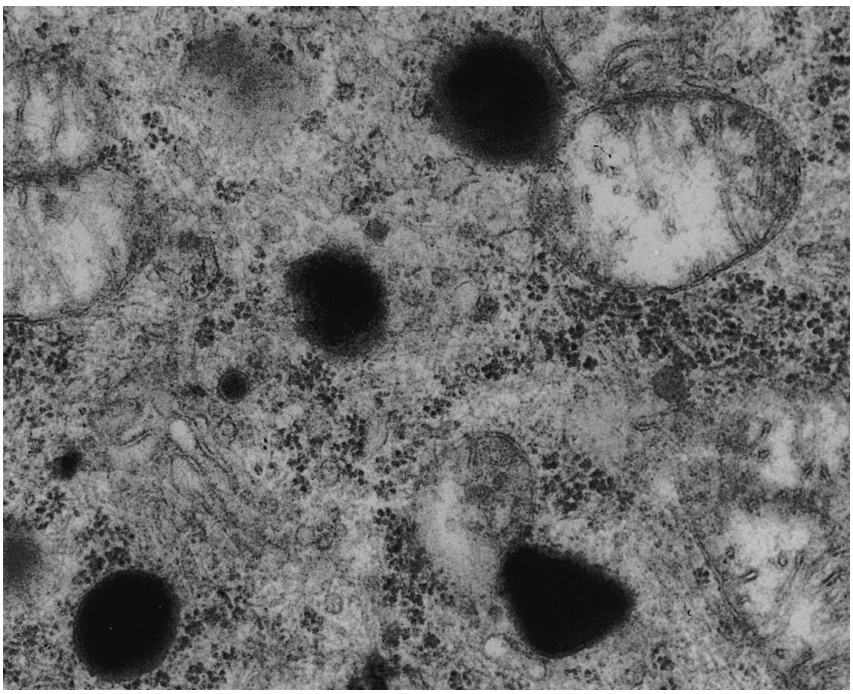

Fig. 2 (continued).

statistical analysis, individual group comparisons were made using a three-way analysis of variance (ANOVA) with repeated measures. The factors of variation were surgery (between subjects), treatment (between subjects) and time (within subjects). Subsequent two-way ANOVAs were carried out as necessary. Individual treatment effects (differences between groups) were analysed using a Newman–Keuls test following significant main effects of treatment by one-way ANOVA. Individual comparisons between sham-operated and autotransplanted groups at each time point were performed by using a Student–Fischer test. The level of significance was $P < 0.05$.

3. Results

The histological study of the spinal cord showed viable clusters of chromaffin cells in arachnoid locations 2 months after the surgical implantation in all the grafted animals examined (Figs. 1 and 2). The chromaffin cells in the cerebrospinal fluid were tenuously attached to the meningeal surfaces, with little apparent integration with the host central nervous parenchyma, as previously reported (Sagen et al., 1986; Sagen and Kemmler, 1989; Sagen and Wang, 1990; Sagen et al., 1990, 1991). The grafts contain connective tissue elements in addition to chromaffin cells. At a higher magnification, chromaffin cells were identified by their characteristic dense granules, and were often seen in the transplant aggregated in a capsule of connective tissue.

3.1. Experiment 1: effects of RB 101 on transplant-induced antinociception

Three-way ANOVA revealed a significant effect of surgery (between subjects, $F(1,48) = 64.50$, $P < 0.0001$), treatment (between subjects, $F(2,48) = 6.01$, $P < 0.005$), and time (within subjects, $F(5,10) = 20.57$, $P < 0.0001$). Significant interactions between surgery and treatment ($F(2,48) = 64.50$, $P < 0.0001$), and treatment and time ($F(10,240) = 64.50$, $P < 0.0001$) were also observed. No significant interactions between surgery and time ($F(5,10) = 1.67$, N.S.), or between surgery, treatment and time ($F(10,240) = 1.01$, N.S.) were shown. Results obtained from subsequent two-way ANOVA (treatment, between subjects; and time, within subjects) are shown in Table 1.

One-way ANOVA at the different time points revealed an effect of treatment in sham-operated animals that was significant 10 min ($F(2,24) = 7.28$, $P < 0.005$) and 20 min ($F(2,24) = 8.63$, $P < 0.005$) after RB 101 administration. In autotransplanted rats, one-way ANOVA revealed a significant effect only 10 min after RB 101 injection ($F(2,24) = 4.71$, $P < 0.05$). Post-hoc comparisons (Newman–Keuls test) showed in sham-operated animals a significant effect of RB 101, at the dose of 5 mg/kg, 10 min ($P < 0.01$) and 20 min ($P < 0.01$) after administration, and at the dose of 1.66 mg/kg, 20 min after injection ($P < 0.01$) (Fig. 3a). In autotransplanted rats, post-hoc comparisons only revealed a significant effect of RB 101, at the dose of 5 mg/kg, 10 min after administration ($P < 0.01$) (Fig. 3b).

Table 1
Summary table of two-way ANOVA between subjects

	Acute treatment	Time	Interaction
Experiment 1			
Sham	$F(2,24) = 8.43,\ P < 0.005$	$F(5,120) = 23.47,\ P < 0.0001$	$F(10,120) = 3.68,\ P < 0.0005$
Transplant	$F(2,24) = 1.66$, N.S.	$F(5,120) = 6.23,\ P < 0.0001$	$F(10,120) = 1.11$, N.S.
Experiment 2			
Sham	$F(3,34) = 1.17$, N.S.	$F(5,115) = 61.97,\ P < 0.0001$	$F(15,170) = 17.77,\ P < 0.0001$
Transplant	$F(3,36) = 2.20$, N.S.	$F(5,180) = 10.79,\ P < 0.0001$	$F(15,180) = 3.38,\ P < 0.0001$

The factors of variation were acute treatment and time.
See Section 2 for details of each experiment.

The nociceptive thresholds obtained at each time point in autotransplanted animals were compared with their respective values obtained in sham-operated animals (one-way ANOVA). Autotransplanted animals treated with saline showed a significant increase in the nociceptive threshold when compared with sham-operated group at all the time points ($P < 0.01$). Autotransplanted animals treated with RB 101 (1.66 and 5 mg/kg) also showed a significant antinociceptive response at all the time points ($P < 0.01$) when compared with control sham-operated animals (Fig. 3).

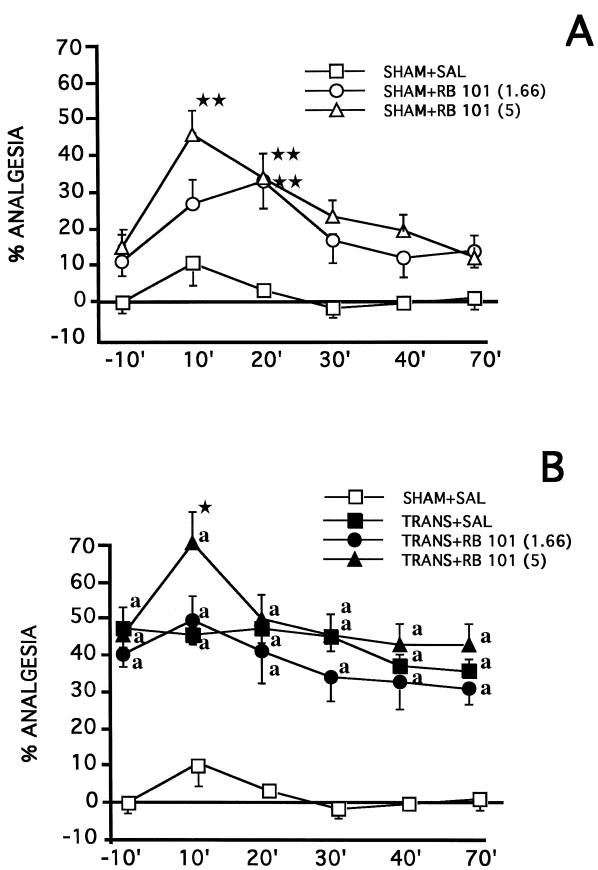

Fig. 3. Effects induced by acute RB 101 administration (1.66 and 5 mg/kg, i.v.) and saline (SAL) in rats sham-operated (SHAM) (A) and autotransplanted (TRANS) (B) in the tail-flick test. Antinociceptive test was performed 28 days after autotransplant surgery. Tail-flick latencies were tested 10 min before and 10, 20, 30, 40 and 70 min after RB 101 administration. Results are expressed as the mean ± S.E.M. of the percentage of change in pain threshold compared to sham-operated saline treated group (time—10 min). $^{\star}P < 0.05$; $^{\star\star}P < 0.01$ vs. saline group receiving the same surgical process. $^{a}P < 0.01$ between autotransplanted groups and sham-operated saline group (Newman–Keuls test).

3.2. Experiment 2: effects of naloxone or phentolamine pretreatment on the responses induced by RB 101 on sham-operated and autotransplanted rats

Three-way ANOVA showed a significant effect of time (within subjects, $F(5,350) = 36.70,\ P < 0.0001$) and surgery (between subjects, $F(1,70) = 10.35,\ P < 0.005$), with no effect of treatment (between subjects, $F(3,70) = 0.96$, N.S.). A significant interaction between time and treatment ($F(15,350) = 10.03,\ P < 0.0005$) was also observed. No significant interactions between time and surgery ($F(5,350) = 2.09$, N.S.), surgery and treatment ($F(3,70) = 2.39$, N.S.), or between surgery, time and treatment ($F(15,350) = 2.29,\ P < 0.005$) were observed. Results obtained from subsequent two-way ANOVA (treatment, between subjects; and time, within subjects) are shown in Table 1.

One-way ANOVA realised at the different time points showed a significant effect in sham-operated rats 10 min ($F(3,34) = 9.71,\ P < 0.0001$) and 20 min ($F(3,34) = 3.71,\ P < 0.05$) after RB 101 administration. This effect was also significant in autotransplanted groups 10 min ($F(3,36) = 2.84,\ P < 0.05$) and 20 min ($F(3,36) = 6.18,\ P < 0.005$) after RB 101 injection. Subsequent Newman–Keuls post-hoc comparisons showed that RB 101 induced a significant antinociceptive effect at the dose of 5 mg/kg in sham-operated rats ($P < 0.01$, 10 min after administration) that was not modified by the administration of phentolamine (10 mg/kg), and was significantly antagonized by the pretreatment with naloxone (1 mg/kg) ($P < 0.01$). The slight antinociceptive response observed 20 min after RB 101 injection was also unaffected by phentolamine (10 mg/kg) and blocked by naloxone (1 mg/kg) pretreatment (Fig. 4). When phentolamine (10 mg/kg) or naloxone (1 mg/kg) were administered alone in sham-operated animals, no modification in the tail-flick latency was observed at any time point (data not shown). In autotransplanted

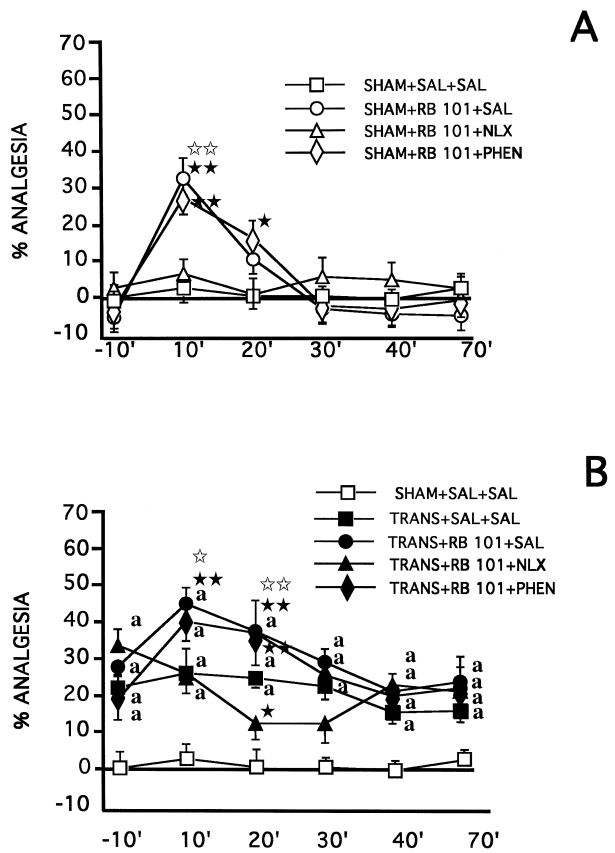

Fig. 4. Effects induced by acute RB 101 administration (5 mg/kg, i.v.) and saline (SAL) in rats sham-operated (SHAM) (A) and autotransplanted (TRANS) (B) in the tail-flick test: influence of naloxone (1 mg/kg, s.c.) (NLX) and phentolamine (10 mg/kg, s.c.) (PHEN) pretreatment. Antinociceptive test was performed 56 days after autotransplant surgery. Tail-flick latencies were tested 10 min before and 10, 20, 30, 40 and 70 min after RB 101 administration. Results are expressed as the mean ± S.E.M. of the percentage of change in pain threshold compared to sham-operated saline treated group (time—10 min). $^{\star}P < 0.05$; $^{\star\star}P < 0.01$ vs. saline group receiving the same surgical process. $^{\star}P < 0.05$; $^{\star\star}P < 0.01$ vs. naloxone group receiving the same surgical process. $^{a}P < 0.01$ between autotransplanted groups and sham-operated saline group (Newman–Keuls test).

rats, the antinociceptive responses induced by RB 101 (5 mg/kg) 10 min ($P < 0.01$) and 20 min ($P < 0.01$) after injection was not modified by phentolamine (10 mg/kg) and reversed by naloxone (1 mg/kg) at both time points ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 4).

The comparison of the nociceptive thresholds obtained at each time point in autotransplanted animals with their respective values obtained in sham-operated rats (one-way ANOVA) revealed a significant antinociceptive effect of the autotransplant in saline-treated groups at all the time points ($P < 0.01$). Autotransplanted animals treated with RB 101 (5 mg/kg) or RB 101 (5 mg/kg) + phentolamine (10 mg/kg) also showed a significantly higher nociceptive threshold than control sham-operated group at all the time points ($P < 0.01$). Autotransplanted group receiving RB 101 (5 mg/kg) + naloxone (1 mg/kg) showed higher tail-flick latencies than control sham-operated animals 10, 40 and 70 min after RB 101 injection, but the response was not significant 20 and 30 min after the administration of the peptidase inhibitor.

4. Discussion

The effects of inhibition of enkephalin catabolism by acute administration of a dual peptidase inhibitor have been investigated on the antinociception induced by adrenal medullary transplantation into the subarachnoid space of the rat spinal cord. The present results confirm that this autotransplantation procedure significantly reduces pain sensitivity in rats, in agreement with previous studies (Sagen et al., 1986; Sagen and Kemmler, 1989; Sagen and Wang, 1990; Sagen et al., 1990, 1991; Ortega-Alvaro et al., 1994, 1997). Long-term transplantation of adrenal medullary tissue has been reported to increase the cerebrospinal fluid concentrations of both endogenous opioids (Sagen and Kemmler, 1989) and biogenic amines (i.e., noradrenaline and serotonin) (Sagen et al., 1991) originating from the transplanted chromaffin cells. The involvement in transplant-induced analgesia of endogenous opioid peptides has been widely demonstrated by the capability of naloxone to block such analgesia (Sagen et al., 1986; Sagen and Wang, 1990; Vaquero et al., 1991). In some of the previous studies, nicotine was used to potentiate the release of neuroactive substances which produce antinociception from the transplanted cells. This procedure was shown to prolong the relief of nociception after transplantations (Sagen et al., 1986; Sagen and Wang, 1990). However, in the present study, the basal release of neuroactive substances from the transplanted cells was presumably sufficient, since an antinociceptive effect in the tail-flick test was observed 28 days after transplantation, and was still significant 56 days after surgery. Therefore, in order to avoid possible interference effects, nicotine stimulation was not used since the aim of this study was to evaluate the modification induced by the peptidase inhibitor, RB 101, on the basal short-lasting analgesia induced after the autotransplant.

One of the most important limitations to the analgesia induced by adrenal medullary transplants is the rapid inactivation of opioid peptides, that are removed from the extracellular space by enzymatic degradation. Acute systemic administration of the complete inhibitor of enkephalin catabolism able to cross the blood–brain barrier, RB 101 (Fournié-Zaluski et al., 1992), induced antinociceptive effects in the tail-flick test in sham-operated rats, as previously reported (Fournié-Zaluski et al., 1992; Noble et al., 1992a). The systemic administration of RB 101 also enhanced significantly the antinociception induced by the autotransplant in this test. This facilitatory effect of RB 101 was observed both 28 and 56 days after surgery. After this acute effect of RB 101, the nociceptive threshold in transplanted animals remained elevated during the whole observation period, such as in transplanted animals receiv-

ing saline. As autotransplanted rats already showed an important antinociceptive response, the absolute increase induced by RB 101 (5 mg/kg) in the percentage of analgesia was slightly lower in this group than in sham-operated rats (see Fig. 3). The antinociceptive response was also stronger in sham-operated animals when RB 101 was administered at a lower dose (1.66 mg/kg). Therefore, the increase in the extracellular levels of endogenous enkephalins produced by the inhibitor of enkephalin catabolism was presumably higher under basal conditions (sham-operated animals) than when the release of these endogenous peptides was already increased by the autotransplantation procedure. As the extracellular level of enkephalins was more elevated in autotransplanted than in sham-operated rats, it was not surprising that RB 101 administration induced a lower, but still significant antinociceptive response in those animals. Previous studies showed that intrathecal administration of kelatorphan, another complete inhibitor of the enkephalin catabolism unable to cross the blood–brain barrier, prolonged the antinociception induced by adrenal medullary transplants after nicotine stimulation (Sagen and Wang, 1990). However, the baseline levels of pain sensitivity in transplanted animals were unaltered by kelatorphan (Sagen and Wang, 1990). The lack of effect of kelatorphan on baseline levels of nociception (Sagen and Wang, 1990) could be due to a lower capability of this peptidase inhibitor to protect endogenous opioids from their catabolism, in agreement with previous results obtained in pharmacological studies in rodents (Fournié-Zaluski et al., 1984, 1992; Noble et al., 1992a). The enhancement of transplant-induced antinociception by RB 101 was observed after systemic administration of the peptidase inhibitor. This ability of RB 101 to cross the blood-brain barrier represents an important advantage with regards to previous compounds in terms of potential clinical application.

The pretreatment with the opioid antagonist, naloxone, but not with the noradrenergic antagonist, phentolamine, antagonized the antinociception induced by RB 101 in both sham-operated and autotransplanted rats. The doses used of naloxone (1 mg/kg) and phentolamine (10 mg/kg) did not induce any intrinsic effect in sham-operated animals (data not shown). This result suggests that the antinociceptive effects induced by RB 101 were due to the inhibition of the degradation of endogenous enkephalins and the consequent enhancement of the extracellular concentration of these endogenous peptides, as previously demonstrated (Bourgoin et al., 1986; Ruiz-Gayo et al., 1992; Daugé et al., 1996). As this antagonism was observed in both sham-operated and autotransplanted animals, it can be hypothesised that the antinociception induced by RB 101 in transplanted animals should be due to inhibition of the degradation of opioid peptides originating from both the host central nervous system and the transplanted chromaffin cells. However, the significant increase of the antinociceptive response induced by RB 101 in transplanted rats and its antagonism by naloxone suggest that opioid peptides released from the transplanted cromaffin cells are important in mediating this response. The administration of naloxone in transplanted rats receiving the peptidase inhibitor reduced the nociceptive threshold to a level significantly lower than in control transplanted animals. Therefore, the release of endogenous opioid peptides from the transplanted cells is high enough to produce a significant antinociceptive response even in the absence of RB 101, as previously shown (Sagen et al., 1986; Sagen and Wang, 1990). However, the lack of effect of phentolamine suggests that catecholamines play only a minor role in the antinociception produced by adrenal medullary transplants. In agreement with these results, we have previously evaluated the effects of naloxone and phentolamine in autotransplanted rats under similar experimental conditions. Naloxone decreased the antinociceptive effects of transplants, whereas phentolamine did not significantly modify the antinociceptive response in autotransplanted rats (Ortega-Alvaro et al., 1994 and unpublished data). Furthermore, other authors have also reported that phentolamine administration only partially decreased the strong antinociceptive response produced after increasing the release of neuroactive substances from the transplanted adrenal medullary cells by injecting nicotine (Sagen et al., 1986). Taken together, these results suggest that transplanted cells presumably release both catecholamines and opioid peptides, but the antinociceptive responses under these experimental conditions seem to be mainly due to opioid peptides. The antinociceptive responses in the tail-flick test were evaluated on days 28 and 56 after surgery, and remained significant at both time points in agreement with previous data (Sagen et al., 1986, 1990), but the antagonists were administered only at the measurements performed on day 56 in order to simplify the experimental design. We cannot exclude that the effects of these antagonists 28 days after transplantations would be different, although this possibility seems unlikely since (1) no histological changes or abnormalities were observed in the cromaffin cells during this period; (2) an important increase of catecholamine levels has been shown by adrenal medullary transplants up to 6 months after the transplantation procedure (Sagen et al., 1991); (3) the increase of endogenous enkephalins produced by such transplantations has been reported during short- and long-term periods (Sagen and Kemmler, 1989).

Several authors have reported the feasibility and efficacy of subarachnoid adrenal medullary transplantation in alleviating terminal cancer pain in humans (Winnie et al., 1993; Pappas et al., 1997). The present results show that administration of inhibitors of enkephalin catabolism able to cross the blood-brain barrier, such as RB 101, may be a useful procedure to enhance the antinociception induced by adrenal medullary transplants. The antinociceptive effects of RB 101 in control animals have been previously compared to those produced by full opioid agonists such as

morphine in several nociceptive models (Noble et al., 1992a; Maldonado et al., 1994; Valverde et al., 1996). When the ED_{50} values could be calculated, the potency of RB 101 was four times lower than morphine on the same nociceptive model (Noble et al., 1992a). Interestingly, chronic administration of RB 101 did not develop tolerance to its antinociceptive effects nor physical dependence, in contrast with morphine treatment under similar experimental condition (Noble et al., 1992b, 1994). Besides, the rewarding effects induced by the repeated administration of RB 101 were less intense than those produced by equianalgesic doses of morphine (Noble et al., 1993; Valverde et al., 1996). The absence of major side-effects suggests a promising antinociceptive profile for these peptidase inhibitors (Roques et al., 1993). The association of adrenal medullary transplantation with the administration of a dual peptidase inhibitor could be particularly interesting considering these weak unwanted side-effects, and the absence of an evident cross-tolerance between adrenal medullary transplants and the analgesia induced by exogenous opiate administration (Wang and Sagen, 1994).

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