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A Calcitonin Gene-Related Peptide Receptor Antagonist Prevents the Development of Tolerance to Spinal Morphine Analgesia

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Tolerance to morphine analgesia is believed to result from a neuronal adaptation produced by continuous drug administration, although the precise mechanisms involved have yet to be established. Recently, we reported selective alterations in rat spinal calcitonin gene-related peptide (CGRP) markers in morphine-tolerant animals. In fact, increases in CGRP-like immunostaining and decrements in specific [^{125}I]hCGRP binding in the superficial laminae of the dorsal horn were correlated with the development of tolerance to the spinal antinociceptive action of morphine. Other spinally located peptides such as substance P, galanin, and neuropeptide Y were unaffected. Thus, the major goal of the present study was to investigate whether the development of tolerance to spinally infused morphine could be modulated by the blockade of dorsal horn CGRP receptors using the potent CGRP antagonist hCGRP$_{8-37}$. Indeed, cotreatments with hCGRP$_{8-37}$ prevented, in a dose-dependent manner, the development of tolerance to morphine-induced analgesia in both the rat tail-flick/tail-immersion and paw-pressure tests. Moreover, alterations in spinal CGRP markers seen in morphine-tolerant animals were not observed after a coadministration of morphine and hCGRP$_{8-37}$. These results demonstrate the existence of specific interaction between CGRP and the development of tolerance to the spinal antinociceptive effects of morphine. They also suggest that CGRP receptor antagonists could become useful adjuncts in the treatment of pain and tolerance to the antinociceptive effects of morphine.

Key words: analgesia; autoradiography; immunostaining; spinal cord; opioids; tolerance

Morphine is used widely in the clinical management of various types of pain, including as an adjunct in the treatment of cancer (Trachtenberg, 1994). Although morphine is very useful as an analgesic, its clinical application in chronic pain is limited by rapid development of tolerance to its antinociceptive properties (Johnstone and Smith, 1992). The basis of tolerance to the antinociceptive actions of morphine and related opioids is only poorly understood (Collier and Schneider, 1969; Yaksh and Noueihed, 1985; Rasmussen et al., 1990; Yamamoto and Meltzer, 1992). For example, although a possible role for brain and spinal cord opioid receptors in tolerance has been proposed (Holt et al., 1975; Pert and Snyder, 1976; Werling et al., 1989), clear evidence for their direct involvement and that of opioid receptor-linked transduction mechanisms (De Vries et al., 1991) is still mostly lacking. Recently it was proposed that tolerance to the antinociception of morphine could be mediated, at least in part, through the activation of physiologically antagonistic systems and/or the inhibition of facilitatory ones (Lake et al., 1991; Trujillo and Akil, 1991; Gutstein and Trujillo, 1993; Rezayat et al., 1994; Stanfa et al., 1994); however, potential antagonistic factors that may be active against tolerance, such as neuropeptide FF (Lake et al., 1991) or cholecystokinin (Rezayat et al., 1994), potentiated morphine analgesia in naive animals (Gouardères et al., 1993b; Rezayat et al., 1994; Stanfa et al., 1994). Thus, recent interest has focused on the possible role of substance P (SP) in the antinociception and tolerance to the spinal effects of morphine, because this neuropeptide is one of the major sensory peptides modulating pain transmission (Henry, 1976). Consistent alterations in spinal SP markers, however, have not been observed in morphine-tolerant animals (Gouardères et al., 1993a; Ménard et al., 1995a).

Calcitonin gene-related peptide (CGRP) is known to be colocalized with SP and glutamate and co-released with SP from primary afferent fibers in the dorsal horn of the spinal cord (Gibson et al., 1984; Woolf and Wiesenfeld-Hallin, 1986). Specific CGRP receptor binding sites are concentrated in the dorsal horn of the spinal cord (Yashpal et al., 1992). Additionally, CGRP induces algesic effects in certain models of nociception (Cridland and Henry, 1988) and modulates acute antinociceptive action of opioid agonists (Welch et al., 1989). In fact, CGRP inhibits the antinociception produced by morphine (Welch et al., 1989), whereas this opiate inhibits the acute release of CGRP in the cord (Pohl et al., 1989). Recently, we reported that increases in CGRP-like immunostaining and reductions in specific [^{125}I]hCGRP binding in the superficial laminae of the dorsal horn were correlated with the development of tolerance to the spinal antinociceptive action of morphine (Ménard et al., 1995a) and [D-Pen^2,D-Pen^5]-enkephalin (Ménard et al., 1995b) acting as μ and δ agonist, respectively. These alterations in spinal CGRP markers were not observed in markers of various other sensory neuropeptides (SP, galanin, neurotensin, and neuropeptide Y). To examine the sig-

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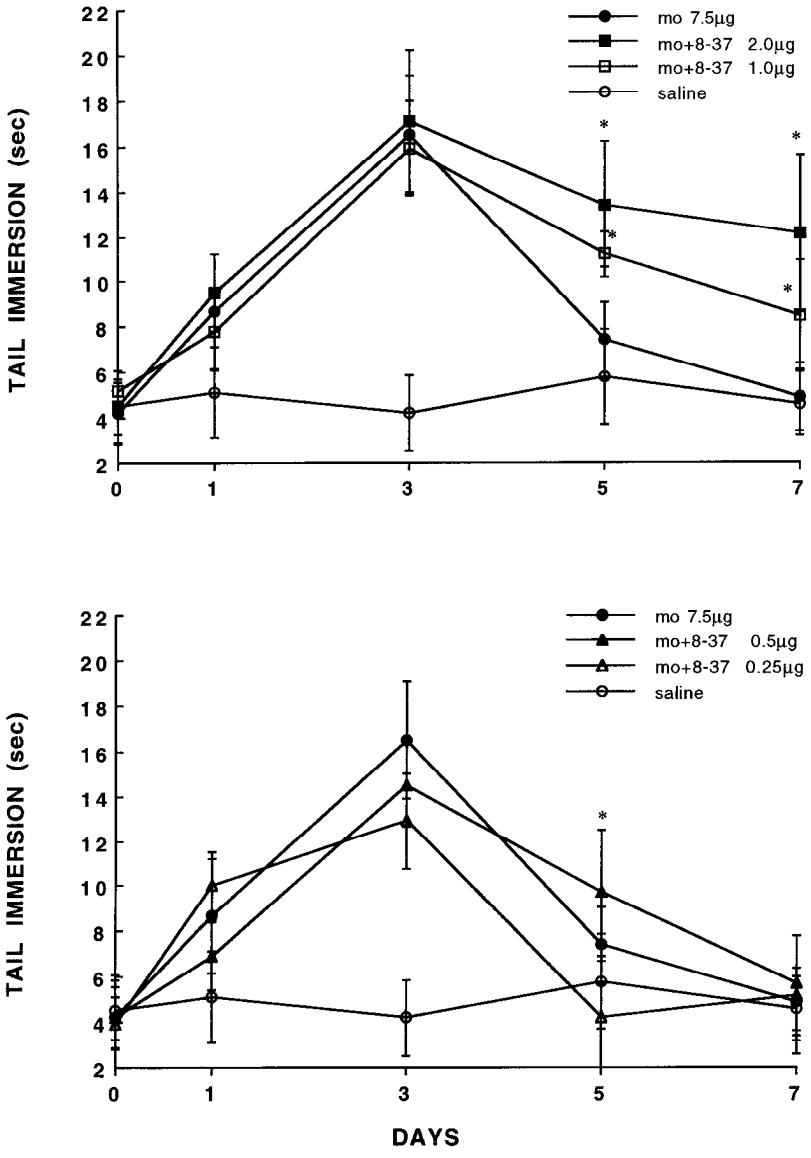

Figure 1. Time course of the antinociceptive effect of a 7 d continuous intrathecal morphine (*mo*: 7.5 μg/hr) infusion alone and with $CGRP_{8-37}$ (*mo + 8–37*: 0.25, 0.50, 1.0, and 2.0 μg/hr) in the tail-immersion test. $CGRP_{8-37}$ at day 5 in the 0.5 μg/hr group and days 5 and 7 in the 1.0 and 2.0 μg/hr groups significantly inhibited the development of tolerance to morphine-induced antinociception. Data shown represent mean ± SEM of 8–12 animals per group. $^*p < 0.05$ indicates a significant difference from corresponding value in the morphine group.

nificance of morphine-induced changes in spinal CGRP systems in the development of tolerance, we have now investigated the action of the CGRP receptor antagonist $CGRP_{8-37}$ (Dennis et al., 1990) on this phenomenon, using a continuous spinal morphine infusion model of tolerance. The potential efficacy of the CGRP blocker was evaluated in antinociceptive tests involving thermal (tail immersion/tail flick) and mechanical (paw pressure) nociceptive stimuli. Our results reveal that $CGRP_{8-37}$ is most effective in preventing the development of tolerance to the analgesic properties of spinally infused morphine.

MATERIALS AND METHODS

Materials. Adult male Sprague–Dawley rats (300–325 gm) obtained from Charles River (St. Constant, Québec, Canada) were used in the study. Animals were maintained according to the guidelines of the Canadian Council on Animal Care and were given free access to food and water. [^{125}I]hCGRP (2000 Ci/mmol), microscales, and Hyperfilms were obtained from Amersham Canada (Oakville, Ontario). Unlabeled hCGRP and $hCGRP_{8-37}$ were synthesized in our laboratories (Institut Nationale de la Recherche Scientifique-Santé, Pointe Claire, Québec, Canada). $hCGRP_{8-37}$ has been characterized extensively as a highly selective, potent, and stable CGRP receptor blocker under various *in vitro* and *in vivo* conditions. It is currently the most potent CGRP antagonist available (Quirion et al., 1992). Polyclonal antiserum to rat CGRP was a generous gift of Dr. J. M. Polak (Royal Postgraduate Medical School, London, UK). Bovine serum albumin (BSA), bacitracin, leupeptin, chymostatin, goat anti-rabbit IgG, peroxidase anti-peroxidase (PAP), 3,3′ diamino benzidine tetrahydrochloride (DAB), and HEPES buffer were obtained from Sigma Chemicals (St. Louis, MO), whereas miniosmotic pumps (model 2001) were purchased from Alzet (Palo Alto, CA). Morphine sulfate was obtained from BDH Pharmaceuticals (Toronto, Ontario, Canada). All other chemicals were of analytical grade and purchased from Fisher Scientific (Montréal, Québec, Canada).

Intrathecal infusion. Under pentobarbital or halothane anesthesia, rats were implanted with indwelling polyethylene catheters (PE-10) as described in detail elsewhere (Gouardères et al., 1993a). For intrathecal infusions, a 7.5 cm catheter was inserted through a slit into the cisternal membrane and advanced gently to reach the lumbar (L_4) subarachnoid space. The catheter was connected previously to a short piece of PE-60 for attachment of the intrathecal catheter to the flow moderator of an Alzet infusion pump. Alzet miniosmotic pumps (model 2001) filled with saline 0.9%, morphine sulfate (7.5 or 10 μg/hr), hCGRP (1.0 μg/hr), $hCGRP_{8-37}$ (0.5 and 1.0 μg/hr), morphine (7.5 μg/hr) plus hCGRP (1.0 μg/hr), or morphine (7.5 or 10 μg/hr) plus $hCGRP_{8-37}$ (0.5–2.0 μg/hr) were implanted subcutaneously, below the back region of the neck, and connected to the intrathecal catheter. Solutions were released continuously at the lumbar level (flow rate, 1 ml/hr) for a 7 d period. Because of the length of the intrathecal catheter, it is well established that significant amounts of morphine will not reach target sites until 25–30 hr after the

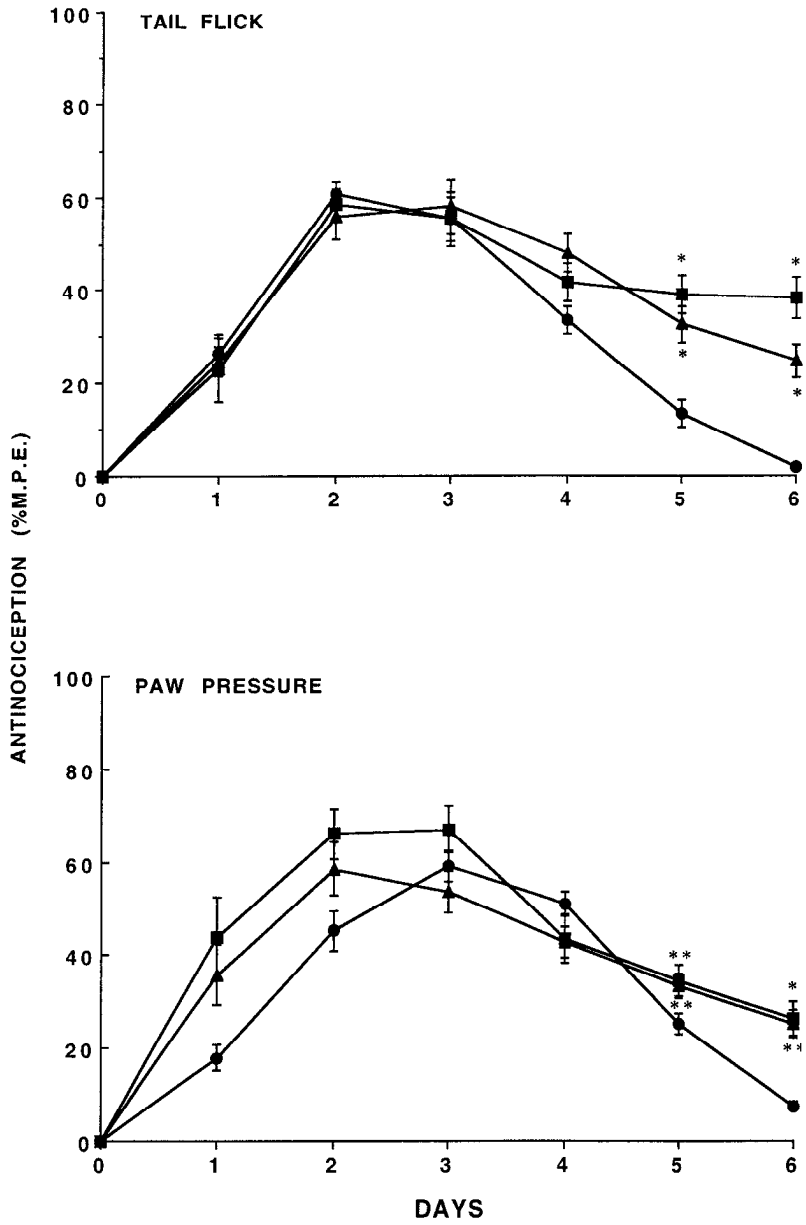

Figure 2. Time course of the antinociceptive effect, expressed as percentage MPE, of a 7 d continuous intrathecal morphine (7.5 μg/hr, ●) infusion alone and with $hCGRP_{8-37}$ (0.5 μg/hr, ▲; 1.0 μg/hr, ■) in the tail-flick (*top*) and paw-pressure (*bottom*) tests. Data shown represent mean ± SEM of four to six animals per group. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant differences from corresponding values in the morphine group.

beginning of the experiments, which explains the delay in attaining the maximal antinociceptive response (see Fig. 1, day 3).

Behavioral evaluation of nociception. For nociceptive studies, animals in the various groups were monitored on a daily basis, using either the tail-immersion/tail-flick (analgesimetric test) or paw-pressure tests.

Tail-immersion test. In the tail-immersion test, the animal held by the experimenter was lowered so that its tail was immersed in a bath of water maintained at a constant temperature of 49°C. The time needed to elicit a nociceptive response (tail flick or whole-body jerk) was noted (cutoff time, 30 sec). At the end of the chronic infusion period, some groups of rats were perfused intracardially or killed by decapitation, and their spinal cords were processed for either CGRP-like immunohistostaining or *in vitro* receptor autoradiography (see below).

Tail-flick test. The tail-flick test (D'Amour and Smith, 1941) was used to evaluate the nociceptive response to a focused thermal stimulus. Radiant heat was applied to the base of tail using an analgesia meter (Owen et al., 1981), with the heat source intensity adjusted to provide a baseline response latency of 2–3 sec and the cutoff time set at 10 sec.

Paw-pressure test. The paw-pressure test used to evaluate the response to a mechanical nociceptive stimulus was a modification of the classic test described by Randall and Selitto (1957). The animal was held gently, and mechanical pressure was applied to the dorsal surface of a noninflamed hindpaw using an air-filled syringe held in an inverted position and connected to a pressure gauge. The pressure in the syringe was gradually increased until a paw-withdrawal response was observed. The pressure was released immediately after this, and the value on the gauge producing response was recorded. In experiments with morphine and CGRP-related peptides, the maximum pressure that elicited withdrawal response was 300 mmHg (cutoff pressure value). In untreated animals, highly reproducible threshold values of pressure were observed when animals were tested on a daily basis.

In the latter two nociceptive tests, baseline response latencies were determined before implantation of minipumps. In implanted animals, antinociception was evaluated once daily between 9 and 10 A.M. The measurements of tail-flick responses were interspersed with those of paw-pressure responses, as previous experiments have demonstrated no interactions between response in these two tests (Loomis et al., 1985). Results at each time point were standardized by expressing values as maximum percentage effect (MPE):

$$\frac{\text{Post drug response} - \text{baseline response}}{\text{Cut-off value} - \text{baseline response}} \times 100.$$

After completion of these experiments, the placement of the catheter in the lumbar region was confirmed by localizing dye injections to this area.

Dose–response experiments. Cumulative dose–response curves for the

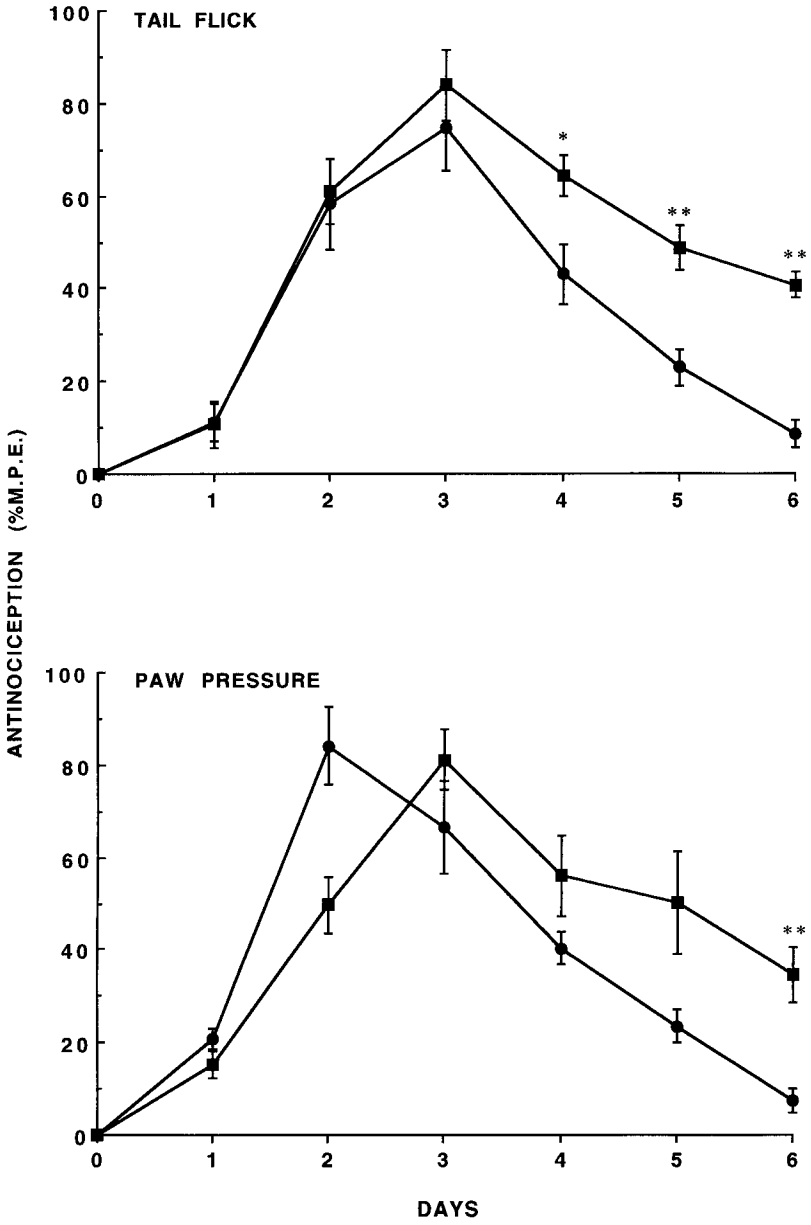

Figure 3. Time course of the antinociceptive effect, expressed as percentage MPE, of a 7 d continuous intrathecal morphine (10.0 μg/hr, ●) infusion alone and with $hCGRP_{8-37}$ (1.0 μg/hr, ■) in the tail-flick (*top*) and paw-pressure (*bottom*) tests. Data shown represent mean ± SEM of five animals per group. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant differences from corresponding values in the morphine group.

antinociceptive action of acute intrathecal morphine in four groups of animals receiving chronic intrathecal infusions were determined using tail-flick and paw-pressure tests. The treatment groups were saline (0.9%, 1 ml/hr, $n = 8$), morphine (7.5 μg/hr, $n = 6$), morphine (same dose) + $CGRP_{8-37}$ (0.5 μg/hr, $n = 7$), and morphine (same dose) + $CGRP_{8-37}$ (1.0 μg/hr, $n = 7$). All animals were tested once daily in both antinociception tests as described above. On day 5 of infusion, the intrathecal infusion catheter was severed from the minipump to allow acute injections. The residual solution present in the infusion catheter was removed by flushing the catheter three times at 2 hr intervals with 5 ml of saline. This procedure avoided the occurrence in morphine-infused rats of intense alladynia when all of the residual morphine was flushed into the intrathecal space. Twenty-four hours after detachment of the catheter, cumulative dose–response curves for acute intrathecal morphine were delivered as described by Mao et al. (1995). Three or four ascending doses of morphine were administered at 30 min intervals to produce gradual antinociceptive responses, which were evaluated 25 min postinjection. Dose–response curves for morphine in each treatment group were constructed, and ED_{50} values for intrathecal morphine action in both tests were determined with the use of a computer program (SIGMOID Version 4, Baker Medical Institute, Melbourne, Australia).

Assessment of motor function. To assess the potential effects of intrathecal infusions on motor function, the animals were tested in an inclined plane test (Rivlin and Tater, 1977), which has been used to evaluate this behavior in rats with spinal cord injury. Each animal used in the tail-flick and paw-pressure tests was placed on an inclined plane, the angle of which was gradually increased from a horizontal to a vertical position. The maximal angle at which the animal maintained its position on the plane was determined. Each animal was assessed daily in this test. The test was conducted in a single-blind fashion such that the assessor was unaware of the treatment received by the animal.

CGRP-like immunostaining. At the end of the 7 d infusion, rats from the saline-treated control group ($n = 6$) and the various experimental groups ($n = 4–6$ per group) used in the tail-immersion test were anesthetized with sodium pentobarbital (65 mg/kg) and perfused intracardially with Bouin's solution, and the lumbar (L4) segment of the spinal cords was dissected out and postfixed in the same fixative for 2 hr. Samples were then cryoprotected, cut serially (20 μm) in the transverse plane, and processed for immunohistochemistry as described in detail elsewhere (Kar et al., 1989). In brief, sections were incubated with polyclonal antisera to rat CGRP (1:2000) (Gibson et al., 1984) at 4°C for 48 hr, washed in PBS, pH 7.4, and incubated for 45 min with goat anti-rabbit IgG (1:25). After washing in PBS, slides were incubated in PAP complex (1:50) for 45 min and then developed in DAB according to the glucose oxidase-nickel enhancement method. The characteristics and specificity of the antiserum used have been described in detail elsewhere

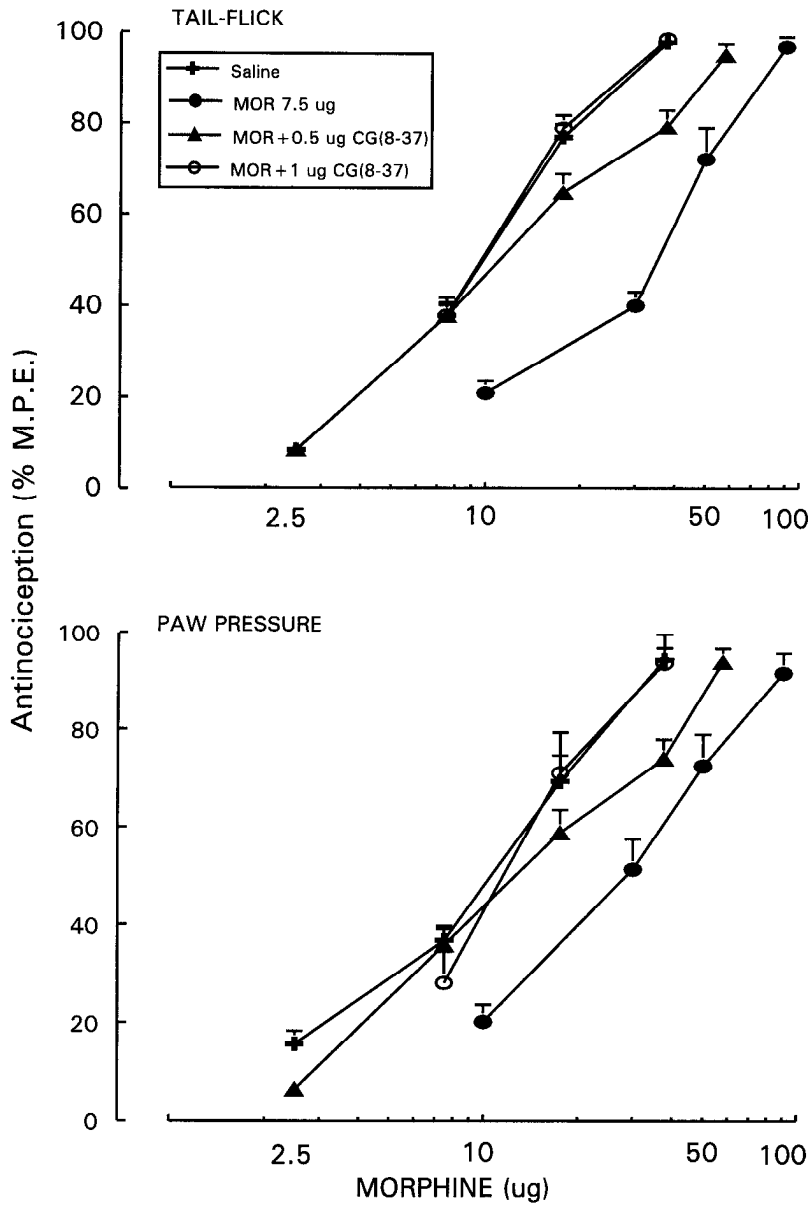

Figure 4. Cumulative dose–response curves for the antinociceptive action of intrathecal morphine in animals after continuous spinal infusion of saline (+), morphine (7.5 μg/hr, ●), and morphine with $CGRP_{8-37}$ (0.5 μg/hr, ▲; 1.0 μg/hr, ○). *Top* and *bottom* depict antinociceptive responses in the tail-flick and paw-pressure tests, respectively. Data shown represent mean ± SEM of six to eight animals. The ED_{50} values obtained from these dose–response curves are represented in Table 1.

(Gibson and Polak, 1986). Sections were then dehydrated in graded alcohols, cleared in xylene, and mounted in Permount before microscopic examination.

In vitro *CGRP receptor autoradiography.* At the end of the 7 d infusion, rats from the saline-treated control group (n = 6) and the various experimental groups (n = 4–6 per group) used in the tail-immersion test were decapitated, and L4 segments of the spinal cords were snap-frozen in 2-methyl butane at −40°C. Tissues were then serially cut (20 μm), thaw-mounted on gelatin-coated slides, and processed for receptor autoradiography using 50 pM [^{125}I]hCGRP as described in detail elsewhere (Ménard et al., 1995a). Briefly, slide-mounted sections were incubated for 90 min at room temperature in HEPES buffer (10 mM, pH 7.4) containing 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1% BSA, 4 μg/ml bacitracin, 4 μg/ml leupeptin, 2 μg/ml chymostatin, and the radioligand. Nonspecific binding was determined in the presence of 1 μm unlabeled hCGRP. Autoradiograms were quantified densitometrically using microscales exposed alongside with radiolabeled sections using an MCID image analysis system (Imaging Research, Ontario, Canada) (Yashpal et al., 1992).

Statistical analysis. Mean (± SEM) of the data are shown in the various figures. Statistical significance was determined using ANOVA. When presented as percentage of control, results were analyzed statistically before transformation of data; p values of <0.05 were used to indicate significant differences between the test group and controls.

RESULTS

Action of $CGRP_{8-37}$ on morphine-induced tolerance to spinal analgesia

Tail immersion test

Figure 1 shows the effects of morphine infusions with and without $hCGRP_{8-37}$ in the tail-immersion test. Morphine (7.5 μg/hr) produced a peak increase in latency to response on day 3. The response returned to baseline value despite continued morphine infusion, reflecting the development of tolerance to the antinociceptive action of morphine. Co-infusion of $hCGRP_{8-37}$ (0.25 to 2.0 μg/hr) with morphine did not alter the peak antinociceptive response but significantly (1.0 and 2.0 μg/hr) delayed its decline, reflecting the inhibition of tolerance to the effect of morphine. In contrast, a co-infusion of CGRP with morphine abolished the morphine response (not shown). Saline (Fig. 1), $CGRP_{8-37}$ (not shown), and hCGRP, when infused alone (not shown), did not influence nociceptive response in this test. After completion of the behavioral test, these groups of animals were perfused or killed to

Table 1. Development of tolerance to morphine

Infusion	Tail flick ED_{50}	Paw pressure ED_{50}
Saline (n = 8)	9.3* (7.9–10.6)	13.1* (7.0–9.2)
$CGRP_{8-37}$ (1.0 μg/hr) (n = 6)	11.6* (6.46–16.8)	18.9* (14.6–23.5)
Morphine (n = 6)	42.8 (36.5–49.1)	35.0 (26.6–43.5)
Morphine + $CGRP_{8-37}$ (0.5 μg/hr) (n = 7)	16.0* (10.7–21.3)	22.4* (11.0–33.7)
Morphine + $CGRP_{8-37}$ (1.0 μg/hr) (n = 7)	9.4* (8.8–9.9)	12.5* (8.6–16.5)

Data represent ED_{50} (95% confidence limit) values for the antinociceptive action of acute intrathecal morphine in animals receiving continuous spinal infusions. Animals were tested in tail-flick and paw-pressure tests 24 hr after termination of the infusion. * $p < 0.05$ indicates significant difference from corresponding value in the morphine-infused group.

determine the status of spinal CGRP markers in the tolerant and nontolerant states (see below).

Tail-flick and paw-pressure tests

The potential of $hCGRP_{8-37}$ to influence morphine antinociceptive action was evaluated further in the continuous infusion model using the tail-flick and paw-pressure tests. The animals were infused with two doses of morphine (7.5 and 10.0 μg/ml/hr) to produce antinociceptive responses of different magnitude.

In animals receiving infusion of the lower dose of morphine (Fig. 2), the antinociceptive response in both tests was submaximal; it peaked at day 3 and declined thereafter to baseline value by day 6. Co-infusion of this dose of morphine with $CGRP_{8-37}$ (0.5 and 1.0 μg/ml/hr) did not alter significantly the peak response to morphine infusion; however, it delayed significantly the decline of response seen in animals receiving morphine alone (Fig. 2*A,B*). Thus, $CGRP_{8-37}$ markedly attenuated the development of tolerance to a submaximal dose of morphine in the tail-flick and paw-pressure tests.

In animals receiving infusion of the higher dose of morphine, the peak antinociceptive in both tests reached a near-maximal value. As in the preceding tests, this response declined after day 3 and returned to baseline value by day 6 (Fig. 3*A,B*). Co-infusion of $hCGRP_{8-37}$ with this dose of morphine did not influence the magnitude of the antinociceptive response in the two tests; however, the decline of the morphine-induced antinociception from peak to baseline level occurring during the 3 d period was slowed significantly by the co-infusion. Thus, $CGRP_{8-37}$ inhibited the development of tolerance to a dose of morphine, producing a near-maximal response.

Dose–response experiments

The cumulative dose–response curves for acute action of morphine after chronic intrathecal infusion of saline, morphine alone, and in combination with two $CGRP_{8-37}$ doses are illustrated in Figure 4. The ED_{50} values for acute intrathecal morphine are represented in Table 1. As shown, intrathecal injection of morphine produced dose-related antinociception in the tail-flick and paw-pressure tests. The dose–response curve for morphine action in animals infused with the opiate for 5 d showed a four- and threefold shift to the right in the tail-flick and paw-pressure tests, respectively. The increase in ED_{50} value in the morphine group is indicative of tolerance to the action of acute morphine. In animals co-infused with morphine and $CGRP_{8-37}$, using the 0.5 and 1.0 μg/hr dose, the dose–response curve for morphine showed a smaller shift or an overlap with the curve obtained in the saline group. The ED_{50} values for morphine action in this group (Table 1) were significantly lower than those obtained in the morphine-infused group, reflecting attenuation or blockade of the development of tolerance by $CGRP_{8-37}$.

Action of $CGRP_{8-37}$ and morphine on motor function

The motor function of animals undergoing morphine infusions with and without $CGRP_{8-37}$ was evaluated in the inclined-plane test to determine whether the tolerant or nontolerant animals exhibited deficits in this function (data not shown). The animals infused with morphine tolerated a slope angle of ~70° in the inclined plane before falling off the plane. During infusion, this value did not vary significantly, regardless of the morphine dose. Animals co-infused with $CGRP_{8-37}$ (0.5 or 1.0 μg/hr) also tolerated a similar angle of slope. The values obtained on successive days after the beginning of the co-infusion were not significantly different from those obtained in the preceding tests. Thus, the motor performance of animals receiving morphine with and without $CGRP_{8-37}$ was not significantly different. Visual examination of fore and hind limbs of animals did not reveal signs of dysfunction. At the completion of these experiments, dye-injection experiments revealed the presence of intrathecal catheter at the lumbar level.

Spinal CGRP-like immunostaining in treated animals

As is already well established (Gibson et al., 1984), CGRP-like immunostaining was found to be concentrated in the superficial laminae of the dorsal horn of the spinal cord (Fig. 5*A*). A marked increase in CGRP-like immunostaining was seen in superficial laminae of the dorsal horn after a 7 d intrathecal infusion with morphine (Fig. 5*B*). This increase was inhibited by a co-infusion with the CGRP antagonist $hCGRP_{8-37}$ (Fig. 5*C*) but not with CGRP itself (Fig. 5*D*). A slight decrease in CGRP-like immunostaining was also observed in laminae I and II of the spinal cord after a 7 d infusion with hCGRP alone (Fig. 5*F*); however, this was not seen after infusion with the antagonist $hCGRP_{8-37}$ (Fig. 5*E*).

Spinal CGRP receptors in treated animals

In accordance with previous results (Sexton et al., 1986; Kruger et al., 1988; Yashpal et al., 1992), specific [^{125}I]hCGRP binding sites in the spinal cord of saline-treated rats are concentrated in the dorsal horn (Fig. 6*A*). The decrease in [^{125}I]hCGRP binding observed in superficial laminae of morphine-treated animals (Figs. 6*B*, 7) was apparently reversed by a co-infusion with $hCGRP_{8-37}$ (Figs. 6*C*, 7) and was enhanced by a co-infusion with hCGRP (Figs. 6*D*, 7). When infused alone, $hCGRP_{8-37}$ failed to have any significant effect on [^{125}I]hCGRP binding (Figs. 6*E*, 7), but under similar conditions hCGRP decreased labeling in all layers, as expected for a full agonist (Figs. 6*F*, 7).

With the focus on substantia gelatinosa and lamina X, the amounts of specific binding for [^{125}I]hCGRP among the saline-treated and the two morphine plus peptide-treated groups were compared. A 7 d treatment with morphine induced a significant decrease in [^{125}I]hCGRP binding in laminae I, II, and III without affecting labeling in lamina X (Fig. 7). A co-infusion with $hCGRP_{8-37}$ reversed the effects of morphine (Fig. 7), whereas a cotreatment with the agonist hCGRP had a somewhat additive (albeit nonsignificant) effect to that of morphine on [^{125}I]hCGRP binding. A 7 d intrathecal infusion with hCGRP alone decreased

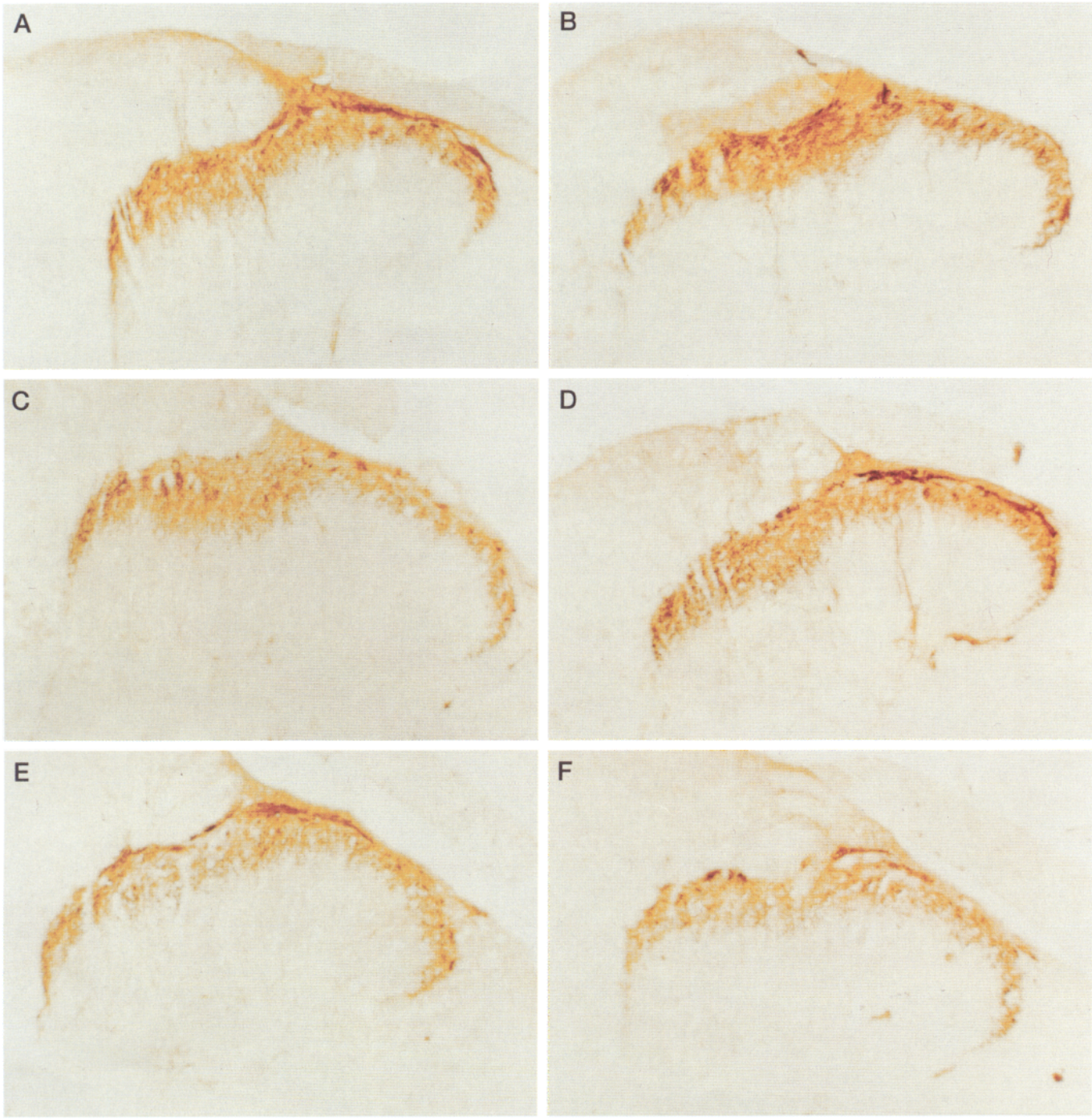

Figure 5. Representative photomicrographs of the distribution of CGRP-like immunohistostaining at the lumbar level (L4) of the spinal cord of rats treated with saline (*A*), morphine (7.5 μg/hr) (*B*), morphine (7.5 μg/hr) plus $hCGRP_{8-37}$ (1.0 μg/hr) (*C*), morphine (7.5 μg/hr) plus hCGRP (1.0 μg/hr) (*D*), $hCGRP_{8-37}$ (1.0 μg/hr) (*E*), and hCGRP (1.0 μg/hr) (*F*) after a 7 d continuous infusion. At least four to six animals per group were studied, all showing the changes depicted in these representative photomicrographs.

[^{125}I]hCGRP binding in laminae I, II, and III, as well as in lamina X, whereas a treatment with $hCGRP_{8-37}$ by itself was devoid of effects on [^{125}I]hCGRP binding (Fig. 7).

DISCUSSION

The present study demonstrates that an intrathecal co-infusion of morphine and $CGRP_{8-37}$, a CGRP receptor antagonist, for 7 d blocks or significantly delays the development of tolerance to the antinociceptive effect of morphine. The inhibition of tolerance to morphine was also reflected in responses obtained with acute intrathecal morphine postinfusion. $CGRP_{8-37}$ reduced or prevented the increase in ED_{50} dose of acute intrathecal morphine produced by the chronic infusion of the opiate for 5 d. These findings were apparent in at least the two types of nociceptive tests involving thermal and mechanical nociceptive stimuli. Thus, the capacity of the CGRP receptor antagonist to attenuate the development of tolerance to morphine analgesia was not dependent on the nature of the applied nociceptive stimuli (heat or pressure).

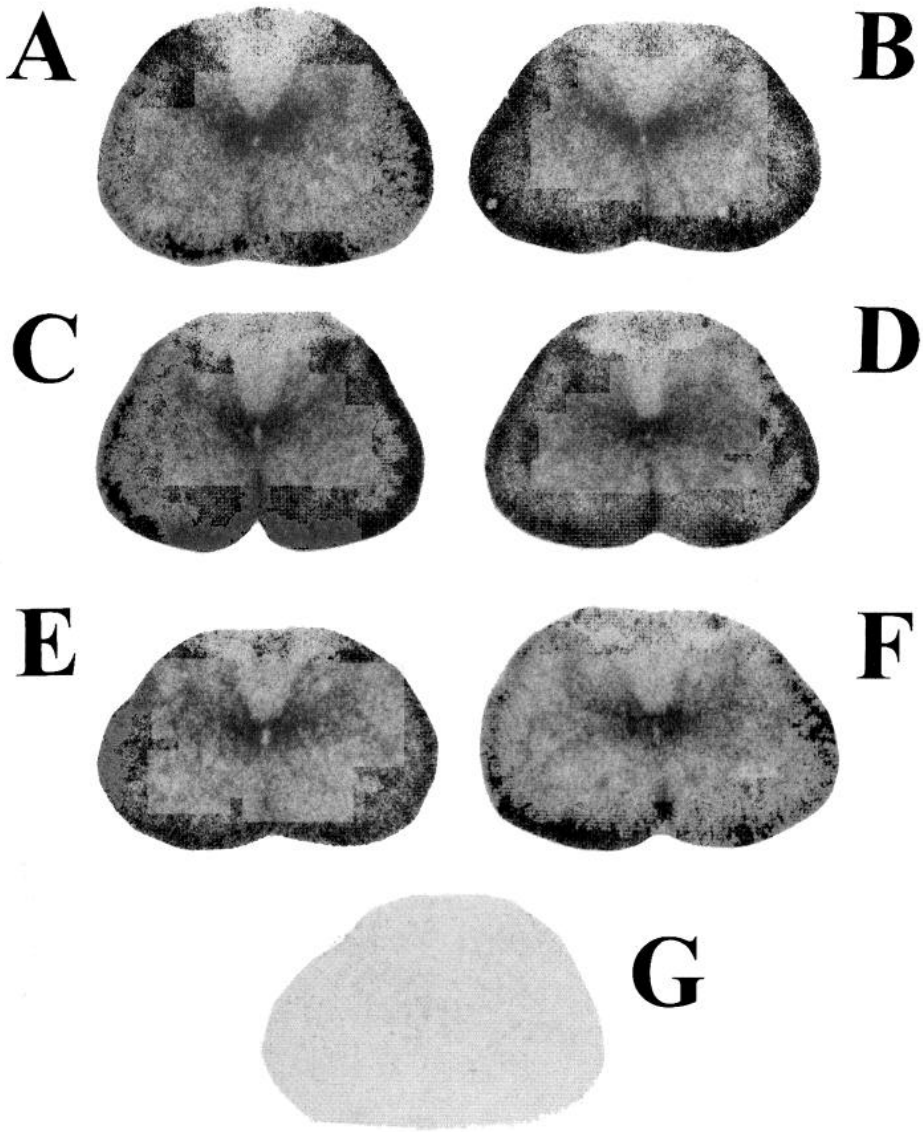

Figure 6. Representative photomicrographs of the autoradiographic distribution of [^{125}I]hCGRP binding sites at the lumbar level (L4) of the spinal cord of rats treated with saline (*A*), morphine (7.5 μg/hr) (*B*), morphine (7.5 μg/hr) plus $hCGRP_{8-37}$ (1.0 μg/hr) (*C*), morphine (7.5 μg/hr) plus hCGRP (1.0 μg/hr) (*D*), $hCGRP_{8-37}$ (1.0 μg/hr) (*E*), and hCGRP (1.0 μg/hr) (*F*) after a 7 d continuous infusion. *G*, [^{125}I]hCGRP binding in the presence of 1 mM hCGRP to determine nonspecific labeling. Although morphine alone decreased specific [^{125}I]hCGRP binding (*A*), a co-infusion with the antagonist $CGRP_{8-37}$ reversed the effects of morphine (*C*), although it was not effective by itself (*E*).

$CGRP_{8-37}$ did not increase the antinociception produced by morphine and did not induce motor deficit, alone or in combination, in the inclined-plane test. The attenuation of tolerance thus was not related to other factors such as an enhancement of morphine response or motor deficits. These observations suggest that $CGRP_{8-37}$ has a highly selective action through which it can inhibit tolerance without enhancing the magnitude of the antinociceptive effects. Interestingly, Yu et al. (1994) demonstrated recently that $hCGRP_{8-37}$ increases paw-withdrawal latency, and a high dose of this peptide (15 μg) attenuated SP-induced hyperalgesia. At the low doses used here, however, $hCGRP_{8-37}$ failed to influence nociception in three different tests over a 6–7 d period. Thus, under our conditions, $CGRP_{8-37}$ is most unlikely to modulate morphine tolerance by itself, influencing nociception in keeping with the usual, rather limited effects of most antagonists (e.g., naloxone, neurokinin blockers) under basal, nonstimulated/unchallenged conditions.

The selective alterations in spinal CGRP-like immunostaining and [^{125}I]hCGRP binding, recently reported to occur after the intrathecal infusion of morphine (Ménard et al., 1995a), were also observed in the present study and were reversed by a cotreatment with $CGRP_{8-37}$. Taken together, these observations support the existence of a unique interaction between the spinal CGRP systems and the development of tolerance to the antinociceptive action of morphine and [D-Pen^2,D-Pen^5]-enkephalin (Menard et al., 1995b). Indeed, we have reported recently that various other peptides known to be present in sensory primary afferent fibers were not altered significantly during the development of tolerance to the spinal antinociceptive properties of morphine. In contrast, spinal CGRP-like immunostaining was enhanced and [^{125}I]hCGRP binding was reduced, changes which paralleled the loss of morphine effect in the tail-immersion test (Menard et al., 1995a). The reversal of these changes by $CGRP_{8-37}$ suggests that alterations in CGRP-like immunostaining and receptor binding contribute to the development of tolerance to morphine analgesia.

Because CGRP is a well known vasodilator substance (Poyner, 1992), it is difficult to exclude fully the participation of the vasculature in the observed effects of $CGRP_{8-37}$ on the development of tolerance to the spinal antinociceptive properties of morphine. The fact that the CGRP antagonist was as effective in two very different pain-related paradigms (tail immersion and paw pressure), however, suggests that a significant involvement of the vasculature is unlikely. Additionally, unlike CGRP, the markers for several other well established vasoactive peptides such as SP, neurotensin, and neuropeptide Y in the dorsal horn were not altered during the development of tolerance to spinal morphine

antinociception, suggesting that involvement of cardiovascular parameters is unlikely to be a major factor in $CGRP_{8-37}$-related blockade of tolerance to morphine.

Modifications in CGRP like-immunostaining and binding in morphine-tolerant animals were restricted to laminae I, II, and III. These regions, among others, are known to be involved in the processing of nociceptive information (Yaksh and Noueihed, 1985). Because morphine-sensitive opioid receptors are located at least partly on primary afferent nerve terminals (Yaksh and Noueihed, 1985; Gouardères et al., 1993a; Arvidsson et al., 1995) and their activation acutely inhibits the release of CGRP (Pohl et al., 1989), the apparent increase in CGRP-like immunostaining in morphine-tolerant animals may be related to the continuous inhibition of its release. This seems rather unlikely, however, as increased receptor densities would be expected under conditions of low transmitter release. In fact, the opposite was observed after morphine treatment, with the densities of [^{125}I]hCGRP binding being reduced in superficial laminae of the tolerant animals. A more likely explanation is that although morphine acutely inhibits CGRP release from primary afferent terminals, this mechanism becomes inoperant in tolerant rats. Thus, the loss of this inhibitory influence of morphine would lead to augmented CGRP synthesis or release leading to a receptor downregulation to avoid overstimulation. Indeed, the observed increase in CGRP-like immunostaining supports this explanation. In the presence of a CGRP receptor antagonist ($hCGRP_{8-37}$), the overactivation and subsequent downregulation of the CGRP receptors presumably would not occur, and a tightly controlled synthesis and release of CGRP from primary sensory afferent fibers would be maintained. $CGRP_{8-37}$ could be reversing alterations induced by chronic morphine in spinal CGRP markers by acting on receptors located either pre- or postsynaptically, the presynaptic ones acting as autoreceptors involved in the regulation of the synthesis and release of CGRP. Experiments are currently in progress to investigate this possibility.

Recently, the administration of NMDA receptor antagonists was reported to block tolerance to morphine analgesia at the spinal level, suggesting a role for glutamate or related transmitters in this phenomenon (Trujillo and Akil, 1991; Gutstein and Trujillo, 1993; Mao et al., 1994). Because CGRP is colocalized with glutamate in primary afferents (DeBiasi and Rustoni, 1988; Merghi et al., 1991), augments the response of dorsal horn neurons to NMDA (Murase et al., 1989), and releases glutamate (Kangrga et al., 1990), the changes in CGRP seen in this study may be mediated by glutamate. The role of glutamate in this regard is unclear, however, because we failed to obtain any evidence for an effect of spinally infused glutamate agonists or antagonists on spinal CGRP markers (our unpublished results) and because NMDA receptor blockers increase morphine catalepsy and lethality (Trujillo and Akil, 1990).

Postreceptor mechanisms involved in the antimorphine tolerance properties of $CGRP_{8-37}$ remain to be established. One possible mechanism by which CGRP could modulate the action of morphine (and of its tolerance) is the activation of the nitric oxide synthase (NOS) pathway in primary afferent terminals. Recent evidence shows that NO can be produced by cultured dorsal root ganglia, and NOS-immunoreactive neurons are present in both neonatal and adult dorsal root ganglia, suggesting that NO likely act as an important signaling molecule in the dorsal horn of the spinal cord (Meller and Gebhart, 1993). In fact, a recent study demonstrated an increase in spinal CGRP release after the administration of sodium nitroprusside, a NO producer (Garry et al., 1994). It has also been proposed that CGRP receptors may be coupled to cGMP production (Poyner, 1992), a second messenger whose levels are elevated by NOS activity (Garthwaite, 1991). It is thus possible that morphine could modulate spinal cord CGRP markers by influencing NO. It could be speculated that tolerance to morphine would increase NO synthesis and alter the release of CGRP from primary afferents. Interestingly, recent data showed that blockers of NO synthesis can retard the development of tolerance to the antinociceptive effects of systemic morphine (Kolesnikov et al., 1992). These findings are rather similar to the effects reported here for $hCGRP_{8-37}$ on spinally infused morphine. Moreover, Kolesnikov et al. (1993) demonstrated that established morphine tolerance could be reversed by inhibition of NO production. Because this reversal phenomenon was very slow in onset, it likely involved several steps subsequent to the modulation of NOS, because the inhibition of this enzyme should have rapidly reinstated the analgesic properties of opioids if NO was the sole mediator involved in tolerance to opioids. Hence CGRP could play a role in that regard, and $hCGRP_{8-37}$, by blocking its receptors, could interfere with the synthesis of NO and restore the antinociceptive properties of morphine.

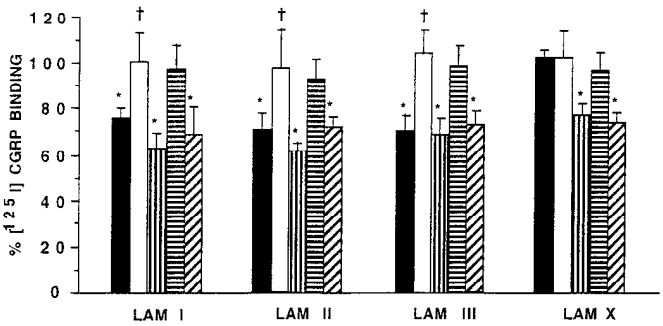

Figure 7. Effects of a 7 d intrathecal infusion of morphine (7.5 μg/hr, *black bars*), morphine (7.5 μg/hr) plus $hCGRP_{8-37}$ (1.0 μg/hr, *open bars*), morphine (7.5 μg/hr) plus hCGRP (1.0 μg/hr, *bars with vertical lines*), $hCGRP_{8-37}$ (1.0 μg/hr, *bars with horizontal lines*), and hCGRP (1.0 μg/hr, *bars with diagonal lines*) on lumbar (L4) [^{125}I]hCGRP binding sites. Data represent the mean ± SEM (n = four to six animals per group). Nonspecific binding in the presence of 1.0 mM hCGRP was subtracted from all readings using computerized densitometry. Statistical significance was evaluated using ANOVA. When presented as percentage of control, results were analyzed statistically before transformation of the data. $^{*}p < 0.05$ from saline-treated rats; $^{\dagger}p < 0.05$ from morphine-treated rats.

In summary, the present data reveal the existence of a novel interaction between CGRP and the development of tolerance to the antinociceptive effects of morphine in the rat spinal cord. The findings in two different pain-related tests that a CGRP receptor antagonist can retard or block the development of tolerance to morphine is likely of significance for a better management of pain in some clinical conditions. In that regard, the design of nonpeptide $CGRP_{8-37}$ homologs is being pursued actively in our laboratories.

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