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Characterization of nociceptin hyperalgesia and allodynia in conscious mice

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1 Intrathecal (i.t.) administration of nociceptin and high doses of morphine induced allodynia in response to innocuous tactile stimuli, and i.t. nociceptin evoked hyperalgesia in response to noxious thermal stimuli in conscious mice. Here we have characterized the nociceptin-induced allodynia and compared it with the morphine-induced allodynia and the nociceptin-evoked hyperalgesia.

2 Nociceptin-induced allodynia was evoked by the first stimulus 5 min after i.t. injection, reached a maximum at 10 min, and continued for a 50 min experimental period. Dose-dependency of the allodynia showed a bell-shaped pattern from 50 pg to 5 ng kg^{-1}, and the maximum effect was observed at 2.5 ng kg^{-1}.

3 Morphine-induced allodynia reached the maximum effect at 15 min and declined progressively until cessation by 40–50 min. The dose-response curve showed a bell-shaped pattern, similar to that induced by nociceptin, with a maximum effect at 0.5 mg kg^{-1}, five orders of magnitude higher than that of nociceptin.

4 The allodynia evoked by nociceptin and morphine were dose-dependently blocked by glycine, D(−)-2-amino-5-phosphonovaleric acid (D-AP5, an *N*-methyl-D-aspartate (NMDA) receptor antagonist), γ-D-glutamylaminomethyl sulphonic acid (GAMS, a non-NMDA receptor antagonist) and methylene blue (a soluble guanylate cyclase inhibitor), but were not affected by muscimol (a γ-aminobutyric $acid_A$ ($GABA_A$) receptor agonist) and baclofen (a $GABA_B$ receptor agonist).

5 Morphine did not inhibit forskolin-stimulated cyclicAMP formation in cultured cells expressing the nociceptin receptor.

6 Nociceptin-induced hyperalgesia was evoked 10–15 min after i.t. injection. Nociceptin produced a monophasic hyperalgesic action over a wide range of doses from 5 fg to 50 ng kg^{-1}. The nociceptin-induced hyperalgesia was blocked by glycine only among the agents examined.

7 None of the pain responses evoked by nociceptin and morphine were blocked by naloxone.

8 These results demonstrate that, whereas the mechanisms of the nociceptin-induced allodynia and hyperalgesia are evidently distinct, they involve a common neurochemical event beginning with the disinhibition of the inhibitory glycinergic response. Morphine may induce allodynia through a pathway common to nociceptin, but the nociceptin receptor does not mediate the action of high doses of morphine.

Keywords: Nociceptin; morphine; allodynia; hyperalgesia; spinal cord; glycine; glutamate

Introduction

Opioid drugs produce their analgesic effects by modulating the ascending and descending pain pathways and the opioid receptors have been the focus of intense research with the hopes of elucidating their roles in pain transmission (Loh & Smith, 1990; Reisine & Bell, 1993; Mansour *et al*., 1995). This has led to the purification of endogenous morphine-like substances from the brain and the identification of three types of receptors, referred to as μ, κ, and δ. Recent cDNA cloning studies have revealed a novel G-protein coupled receptor with a high degree of amino acid sequence homology to the previously cloned μ, κ and δ-opioid receptors. This opioid receptor homologue, designated as ORL_1 in man (Mollereau *et al*., 1994) and its rat counterparts ROR-C (Fukuda *et al*., 1994) and LC132 (Bunzow *et al*., 1994) did not show high affinities for opioid ligands. Recently, purification and characterization of the heptadecapeptide called nociceptin/orphanin FQ (hereafter nociceptin) have been succeeded as an endogenous ligand of this receptor (Meunier *et al*., 1995; Reinscheid *et al*., 1995; Okuda-Ashitaka *et al*., 1996). Although all opioid receptors and the opioid receptor homologue ROR-C mediate inhibition of forskolin-induced adenosine 3′:5′-cyclic monophosphate (cyclicAMP) accumulation and show a similar localization in the spinal cord (Reisine & Bell, 1993; Bunzow *et al*., 1994; Mollereau *et al*., 1994), nociceptin differs from other endogenous opioids which produce analgesic effects. Intrathecal (i.t.) administration of nociceptin induces a hyperalgesic response to noxious stimuli (Reinscheid *et al*., 1995) and an allodynic response to innocuous tactile stimuli (Okuda-Ashitaka *et al*., 1996).

Because of its potent analgesic effect, morphine is widely used in the clinical management of pain, but its use is often accompanied by tolerance and dependency. This has led to the use of high doses of the opiate and it has shown various side effects such as myoclonic seizures, ventilatory depression, urinary retention, and pruritis. It has also been found that i.t. administration of high doses of morphine, far higher than that required for analgesia, produce a periodic spontaneous agitation and evoke an allodynic response to innocuous tactile stimuli in rats (Woolf, 1981; Yaksh *et al*., 1986; Yaksh & Harty, 1988; Frenk *et al*., 1988). Similar observations were obtained in man following administration of high i.t. doses of morphine (Stillman *et al*., 1987) Unlike the analgesic action of morphine mediated by the μ-opioid receptor, allodynic responses showed no stereoselectivity towards opioids and were not reversed by naloxone, suggesting that this excitation induced by i.t. mor-

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phine is not an opioid receptor-mediated event (Yaksh *et al*., 1986; Yaksh & Harty, 1988). This prompted us to examine whether the nociceptin receptor mediates the action of high doses of morphine.

The dorsal horn of the spinal cord is an important site for pain transmission and many neurotransmitters are involved in the modulation of incoming pain information (Yaksh & Aimone, 1989). It has become clear that increased sensitivity to noxious and non-noxious stimuli may involve long-term functional changes in the spinal cord, as evidenced by increased postsynaptic neurone excitability and responsiveness, and morphological or functional modifications of the afferent terminal (Woolf, 1994). A growing body of evidence suggests that the two major neurotransmitter systems, inhibitory and excitatory amino acids, can significantly modulate sensory processing in the dorsal horn. Glycine and γ-aminobutyric acid (GABA) are both inhibitory neurotransmitters that mediate fast synaptic inhibition in the nervous system. On the other hand, the primary afferent fibres have glutamate as their principal excitatory neurotransmitter. This receptor family is classified into three groups, the *N*-methyl-D-aspartate (NMDA), non-NMDA (AMPA-kainate), and metabotropic glutamate (mGluR) receptors (Monaghan *et al*., 1989; Hollmann & Heinemann, 1994). Morphine is known to inhibit the release of excitatory and inhibitory amino acids in the spinal cord (Dostrovsky & Pomeranz, 1973; Davies & Duggan, 1974; Werz & MacDonald, 1982). Although the mechanism of action underlying the excitatory effects of i.t. morphine is not fully understood, the morphine-induced allodynia has been suggested to be exerted through anti-glycinergic and/or anti-GABAergic effects (Yaksh, 1989). In order to elucidate the cellular mechanism(s) of nociceptin in the pain transmission at the spinal cord level, we have studied the interactions of nociceptin with other neurotransmitters in nociceptin-evoked allodynia and hyperalgesia. Furthermore, we have characterized the morphine-induced allodynia and compared the mechanisms involved in nociceptin-induced allodynia with those involved in morphine-induced allodynia.

Methods

Intrathecal administration

Male ddY mice weighing 22 ± 2 g were used in this study. The animals were housed under conditions of a 12 h light-dark cycle, a constant temperature of $22 \pm 2^{\circ}$C and $60 \pm 10\%$ humidity. A 27-gauge stainless-steel needle (0.35 mm, o.d.) attached to a microsyringe was inserted between the L_5 and L_6 vertebrae by a slight modification of the method of Hylden & Wilcox (1980). Drugs in vehicle were injected slowly into the subarachnoid space of conscious mice. It was previously confirmed by use of Coomassie brilliant blue that the injected solution does not extend to the cervical segments (Uda *et al*., 1990).

Studies on allodynia

Studies on allodynia were carried out essentially according to the method of Yaksh & Harty (1988). The mice were divided into groups ($n = 6$/group). Drug-treatment groups were injected with 5 μl of vehicle containing various doses of test agents. Control mice were given physiological saline (5 μl). After the i.t. injection, each mouse was placed in an individual $13 \times 8.5 \times 13$ cm Plexiglas enclosure with wood chips on the floor and observed. Mechanical allodynia was assessed once every 5 min over a 50 min period by light stroking of the flank of the mice with a paintbrush. The allodynic response was ranked as follows: 0, no response; 1, mild squeaking with attempts to move away from the stroking probe; 2, vigorous squeaking evoked by the stroking probe, biting at the probe or strong efforts to escape. Thus, the maximum possible cumulative scores for allodynia of 6 mice are $2 \times 6 = 12$ in any 5 min period and $2 \times 6 \times 10 = 120$ over a 50 min period, respectively, and this was taken as 100%. To evaluate the effect of agents on allodynia, we assessed the effect at the maximal score of allodynia obtained 10 min after i.t. injection of 2.5 ng kg^{-1} nociceptin and 15 min after i.t. injection of 0.5 mg kg^{-1} morphine, respectively. The animals were used only for one experiment.

Hot plate test

Mice were placed on a hot plate maintained at 55°C, and the elapsed time until the mice showed the first avoidance responses (licking the feet, jumping or rapidly stamping the paws) was recorded as described by Woolfe & MacDonald (1944). The response time of the mice to the hot plate was measured at given times after i.t. injection. The animals were used only for one measurement in each experiment.

Studies on allodynia and hyperalgesia were conducted with the approval of the local ethics committee and in accordance with the guidelines of the Ethics Committee of the International Association for the Study of Pain (Zimmermann, 1983).

Chemicals

Nociceptin was a generous gift from Dr Shinro Tachibana of Eisai Co., Tsukuba, Japan. Morphine hydrochloride was obtained from Takeda Chemical Ind., Ltd. (Osaka, Japan). L(+)-2-Amino-4-phosphonobutanoic acid (L-AP4), D(−)-2-amino-5-phosphonovaleric acid (D-AP5), and γ-D-glutamylaminomethyl sulphonic acid (GAMS) were obtained from Cambridge Research Biochemicals (Cambridge, U.K.). Cyclic aminoheptanoyl-Phe-D-Trp-Lys-(OBz)-Thr (PSOM160) was supplied by Bachem Fine Chemicals (Torrance, CA, U.S.A.). Baclofen, L-glycine, muscimol, naloxone, N^{ω}-nitro-L-arginine methyl ester (L-NAME), and taurine were purchased from Sigma (St. Louis, MO, U.S.A.). Haemoglobin (Hb) was obtained from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.). All other chemicals were of reagent grade. All drugs were dissolved in sterile saline on the day of experiments and kept on ice until used. All drugs including saline were coded to assure blind testing.

Cyclic AMP assay

The cyclicAMP contents in Chinese hamster ovarian (CHO) cells expressing the nociceptin receptor (CHO-ROR-C cells) were determined by radioimmunoassay with an Amersham cyclicAMP assay kit as described previously (Okuda-Ashitaka *et al*., 1996). Briefly, CHO-ROR-C cells cultured in 24-well plates (2×10^5 cells/assay) were preincubated for 5 min with 0.9 ml of HEPES-buffered saline solution (composition, mM: NaCl 125, KCl 4.7, $CaCl_2$ 2.2, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 15, glucose 11 and HEPES 15, pH 7.4) supplemented with 0.5 mM 3-isobutyl-1-methylxanthine. Reactions were started by addition of 3 μM forskolin and various concentrations of nociceptin or morphine (0.1 ml). After 10 min incubation at 37°C, reactions were terminated by removal of media and the addition of ice-cold 5% trichloric acid solution (1 ml). The trichloric acid extracts were subjected to cyclicAMP assays.

Statistics

Data were analysed by parametric or non-parametric ANOVA. Statistical significance ($P < 0.05$) was further examined by Duncan's test or Williams' test for multiple comparison. IC_{50} values with 95% confidence limits (95% C.L.) were calculated by use of the computer programme of Probit test.

Results

Effects of i.t. nociceptin and morphine on pain responses

The i.t. administration of nociceptin or morphine hydrochloride resulted in prominent agitation responses, such as

vocalization, biting and escape from the probe, to tactile stimuli applied to the flank. Brushing of the face or tactile stimulation of the forepaws did not give any response, indicating that the allodynia appeared limited to the caudal dermatomes of the body. The i.t. administration of saline in conscious mice had no effect on allodynia. Figure 1a presents the time courses of allodynia evoked by nociceptin 0.05, 0.25 and 2.5 ng kg^{-1}. Nociceptin-induced allodynia showed similar time courses for the first 15 min over a wide range of doses; the allodynia was evoked by the first stimulus 5 min after i.t. injection and the maximum effect was observed at 10 min. The response to nociceptin at 2.5 ng kg^{-1} was long lasting and did not disappear by 50 min. The allodynic score was $66.7 \pm 16.7\%$ of the maximum possible cumulative score even at 50 min.

Figure 1b presents the time courses of allodynia evoked by morphine 0.5, 1 and 2 mg kg^{-1}. Morphine-induced allodynia was evoked by the first stimulus 5 min after i.t. injection, reached a maximum at 15 min and then declined progressively with decreased intensity until cessation at 40–50 min. As compared with the allodynia induced by nociceptin (Figure 1a), the response was weak and disappeared by 50 min.

Intrathecal nociceptin, but not morphine, induced hyperalgesia as assessed by the hot plate (55°C) test. There was no significant difference in the latency period between mice not given an i.t. injection (14.4 ± 0.4 s, mean ± s.e.mean, $n = 10$) and those injected (i.t.) with saline (14.0 ± 0.5 s, at 15 min) over the 60 min experimental period. Intrathecal administration of 50 pg kg^{-1} nociceptin shortened the response latency to 7.9 ± 0.6 s at 15 min after i.t. injection, demonstrating that nociceptin (i.t.) induced mice to develop increased sensitivity to the thermal stimuli. Nociceptin-induced hyperalgesia was evoked 10 min after i.t. injection and the maximum effect was observed at 15 min. The hyperalgesic response was long lasting and had not disappeared by 60 min at higher doses of nociceptin (Figure 1c).

Figure 2a shows dose-dependence of the hyperalgesia and allodynia induced by nociceptin. The hyperalgesic effect of nociceptin was assessed 15 min after i.t. injection. Nociceptin produced a monophasic hyperalgesic action over a wide range of doses from 5 fg to 50 ng kg^{-1}. On the other hand, allodynic scores obtained for the overall 50 min experimental period were accumulated and expressed as a % of the maximum possible score. In contrast to hyperalgesia, dose-dependency of nociceptin-induced allodynia showed a bell-shaped pattern. The allodynia was observed at a dose as low as 50 pg kg^{-1} nociceptin and the maximal effect (75.8% of the maximum possible cumulative score over the 50 min experimental period) was observed at 2.5 ng kg^{-1}.

The morphine-induced allodynia showed similar time courses at higher doses of morphine. The dose-dependency of morphine-induced allodynia showed a bell-shaped pattern in the dose-range 0.25 to 2.5 mg kg^{-1}, five orders higher than that of nociceptin-induced allodynia, and the maximal effect observed at 0.5 mg kg^{-1} was 49.2% of the maximum possible cumulative score.

Effects of glycine and GABA receptor agonists on nociceptin- and morphine-evoked allodynia

In order to elucidate the pathway(s) of the nociceptin-induced allodynia and to compare it with that of the morphine-induced one, we first investigated the involvement of glycine and GABA receptors in the nociceptin-induced allodynia by use of glycine, taurine (a glycine receptor agonist), muscimol (a $GABA_A$ receptor agonist), and baclofen (a $GABA_B$ receptor agonist). The effects of the agents were evaluated by the maximum value of the nociceptin-induced allodynia obtained 10 min after injection of 2.5 ng kg^{-1} nociceptin. The score of allodynia was 83.3% of the maximum possible score and this was taken as 100%. As shown in Figure 3a, the allodynia evoked by nociceptin was dose-dependently blocked by glycine with an IC_{50} value (95% C.L.) of 16.1 ng kg^{-1} (5.16–232.0 ng kg^{-1}). In contrast, the nociceptin-induced allodynia was not affected by either taurine, muscimol or baclofen at doses up to 50, 5.0 and 5.0 μg kg^{-1}, respectively.

We next investigated the involvement of glycine and GABA receptors in morphine-induced allodynia. The score of allodynia induced by 0.5 mg kg^{-1} morphine alone was 83.3% of the maximum possible score at 15 min and this was taken as 100%. As shown in Figure 3b, taurine and glycine dose-dependently blocked the morphine-induced allodynia with IC_{50} values (95% C.L.) of 1.96 μg kg^{-1} (168 ng kg^{-1}–34.8 μg kg^{-1}) and 89.5 ng kg^{-1} (3.10 ng kg^{-1}–17.3 μg kg^{-1}), respectively. But the allodynia was not antagonized by either muscimol or baclofen.

Effects of glutamate receptor antagonists on nociceptin- and morphine-evoked allodynia

We investigated the involvement of glutamate receptors in the allodynia by use of D-AP5 (an NMDA receptor antagonist), GAMS (a non-NMDA receptor antagonist), and L-AP4 (a mGluR antagonist). The allodynia caused by nociceptin was dose-dependently blocked by D-AP5 and GAMS with IC_{50} values (95% C.L.) of 3.68 μg kg^{-1} (0.45–13.77 μg kg^{-1}) and 5.45 μg kg^{-1} (14.2 ng kg^{-1}–60.92 μg kg^{-1}), respectively, but was not affected by L-AP4 at doses up to 50 μg kg^{-1} (Figure 4a).

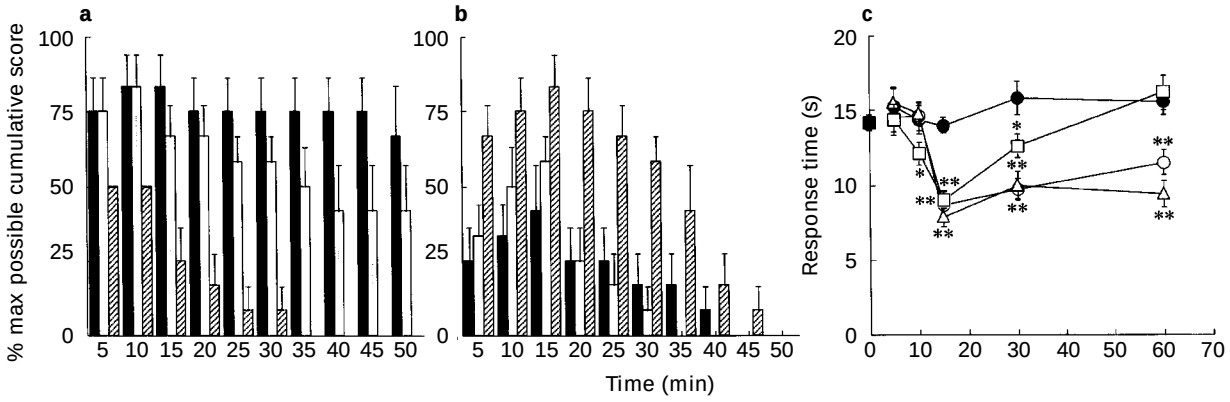

Figure 1 Time courses of allodynia (a, b) and hyperalgesia (c). (a) Mice were injected i.t. with 50 pg kg^{-1} (hatched columns), 0.25 ng kg^{-1} (open columns) and 2.5 ng kg^{-1} (solid columns) nociceptin. (b) Mice were injected i.t. with 0.5 mg kg^{-1} (hatched columns), 1 mg kg^{-1} (open columns), and 2 mg kg^{-1} (solid columns) morphine. Assessment of allodynia was made as described in Methods. Each column (mean ± s.e.mean) represents % of the maximum possible cumulative score (= 12) of six mice evaluated every 5 min. (c) Mice were injected i.t. with 5 fg kg^{-1} (□), 50 pg kg^{-1} (○) and 5 ng kg^{-1} (△) of nociceptin or saline (●) and were not treated (■). The time until the mice showed the first avoidance response to the hot plate (55°C) was measured at the indicated times after the injection. Each point represents the mean and vertical lines show s.e.mean ($n = 10$). Statistical analyses were carried out by Duncan's test. $^*P < 0.05$, $^{**}P < 0.01$, compared with the saline-injected group at the respective time point.

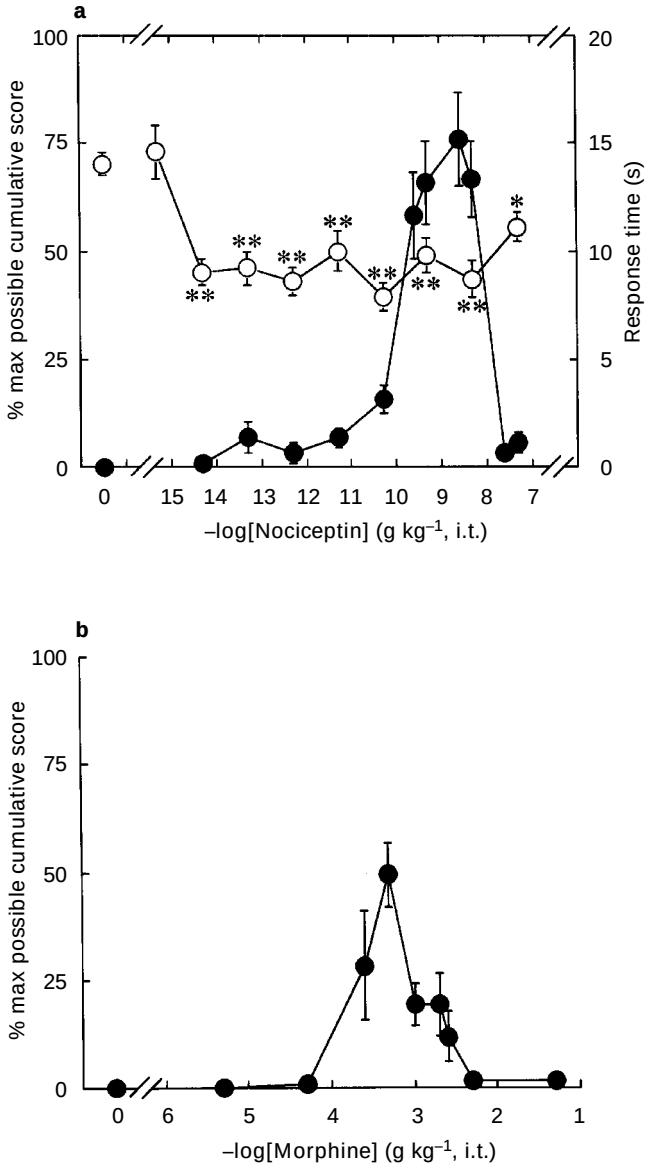

Figure 2 Dose-dependence of the allodynia and hyperalgesia effects of i.t. nociceptin and morphine. Mice were injected (i.t.) with various doses of nociceptin (a) and morphine (b). Allodynia (●) was assessed every 5 min for 50 min after injection as described in Methods. The values (mean ± s.e.mean (vertical lines), $n=6$) of allodynia shown are expressed as % of the maximum possible cumulative score (=120) over the 50 min period. For hyperalgesia (○), the time until the mice showed the first avoidance response to the hot plate (55°C) was measured 15 min after the i.t. injection. Each point represents the mean and vertical lines show s.e.mean ($n=10$). Statistical analyses were carried out by Duncan's test. $^*P<0.05$, $^{**}P<0.01$, compared with saline-injected group (14.0 ± 0.5 s).

As shown in Figure 4b, D-AP5 and GAMS dose-dependently blocked the morphine-induced allodynia with IC_{50} values (95% C.L.) of 13.8 μg kg^{-1} (1.07–219 μg kg^{-1}) and 25.1 μg kg^{-1} (3.87–243 μg kg^{-1}), respectively. These values are 4 to 5 fold higher than those needed to block the nociceptin-induced allodynia (Figure 4a). Similarly, the morphine-induced allodynia was not antagonized by L-AP4.

Involvement of nitric oxide (NO) in nociceptin- and morphine-evoked allodynia

To clarify the involvement of the NO system in allodynia induced by nociceptin and morphine, we further examined the effect of the NO synthase inhibitor L-NAME, the NO scavenger Hb, and the soluble guanylate cyclase inhibitor methylene blue (MB) on the allodynia. As shown in Figure 5a, the allodynia induced by nociceptin was dose-dependently blocked by MB with an IC_{50} value (95% C.L.) of 2.64 ng kg^{-1} (0.57–12.73 ng kg^{-1}) and 5.0 mg kg^{-1} Hb, but was not affected by L-NAME at doses up to 50 ng kg^{-1}.

On the other hand, the morphine-induced allodynia was dose-dependently blocked by both L-NAME and MB with IC_{50} values (95% C.L.) of 1.97 ng kg^{-1} (0.50–7.79 ng kg^{-1}) and 13.8 ng kg^{-1} (3.02–259 ng kg^{-1}), respectively (Figure 5b).

The lack of involvement of the nociceptin receptor in morphine-evoked allodynia

We examined whether nociceptin receptors were involved in allodynia induced by high doses of morphine. Because of the lack of selective antagonists for the nociceptin receptor, at present, we examined the inhibitory activity of morphine on forskolin-induced cyclicAMP accumulation in CHO-ROR-C cells. As shown in Figure 6, whereas nociceptin inhibited forskolin-induced cyclicAMP formation in CHO-ROR-C cells in a concentration-dependent manner with an IC_{50} value of 10 pM, morphine did not inhibit it, but rather stimulated it at concentrations higher than 10 μM.

We investigated the involvement of somatostatin and the opioid receptors in the morphine-induced allodynia by simultaneous injection of the somatostatin antagonist, PSOM160, (Ohkubo *et al*., 1990) and naloxone. Neither PSOM160 nor naloxone blocked the morphine-induced allodynia at doses up to 50 and 500 μg kg^{-1}, respectively (data not shown).

Effect of various agents on nociceptin-evoked hyperalgesia

To compare the involvement of neurotransmitter systems in the nociceptin-induced allodynia and hyperalgesia, we examined the effect of various agents on the hyperalgesia. The effect of the agents on the hyperalgesia was evaluated by studying the maximum effect obtained 15 min after injection of 50 pg kg^{-1} nociceptin. The nociceptin-induced hyperalgesia was blocked by glycine, but not by either muscimol, baclofen or the glutamate receptor antagonists, D-AP5, GAMS, L-AP4, or agents affecting the NO system, L-NAME, Hb and MB (Figure 7). The hyperalgesia evoked by nociceptin was not affected by naloxone at 50 μg kg^{-1} (data not shown).

Discussion

It has recently been shown that i.t. injection of nociceptin induces allodynia in conscious mice (Okuda-Ashitaka *et al*., 1996) and hyperalgesia by the tail-flick test (Reinscheid *et al*., 1995). In the present study, we have extended previous studies and characterized the nociceptin-induced allodynic response to innocuous tactile stimuli and hyperalgesia by the hot plate test. Nociceptin-induced allodynia and hyperalgesia were blocked by simultaneous injection of the inhibitory amino acid glycine, but not of the $GABA_A$ agonist, muscimol and the $GABA_B$ agonist, baclofen (Figures 3a and 7). These results suggest that the mechanisms of nociceptin-induced allodynia and hyperalgesia involve a common neurochemical event beginning with the disinhibition of the inhibitory glycinergic response, not of the GABAergic response, by presynaptic depression of glycine release or by modulation of inhibitory amino acid responsiveness. Antagonists for NMDA and non-NMDA receptors, but not the mGluR antagonist, blocked the nociceptin-induced allodynia (Figure 4a). This rules out the possibility that glycine acts through the glycine regulatory site of the NMDA receptor channel. Consistent with these results, we have recently demonstrated that i.t. administration of the convulsive alkaloid strychnine (a strychnine-sensitive glycine receptor antagonist) induced allodynia by activation of NMDA and non-NMDA

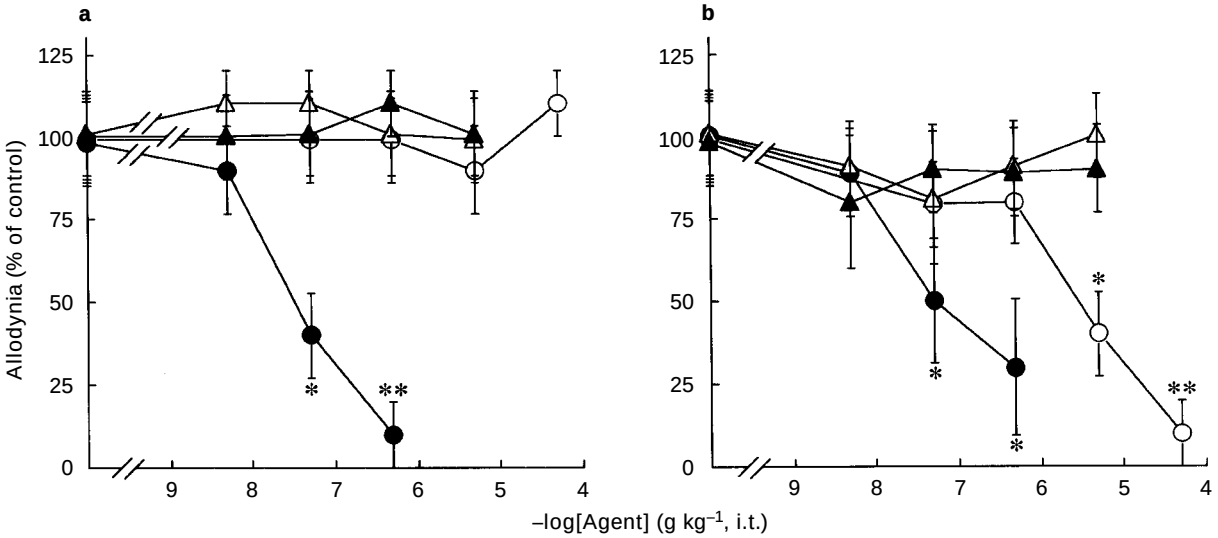

Figure 3 Effect of glycine and GABA receptor agonists on allodynia. Nociceptin (a, 2.5 ng kg^{-1}) or morphine (b, 0.5 mg kg^{-1}) was injected simultaneously with various doses of taurine (○), glycine (●), muscimol (△) or baclofen (▲) into the subarachnoid space. Allodynia was assessed 10 min and 15 min after injection of nociceptin and morphine, respectively. The allodynic scores of nociceptin and morphine alone were the same, 83.3±10.5% of the maximum possible cumulative score and taken as 100%. The values shown are the mean and vertical lines s.e.mean ($n=6$). Statistical analyses were carried out by Williams' test. *$P<0.05$, **$P<0.01$, compared with nociceptin- or morphine-injected group.

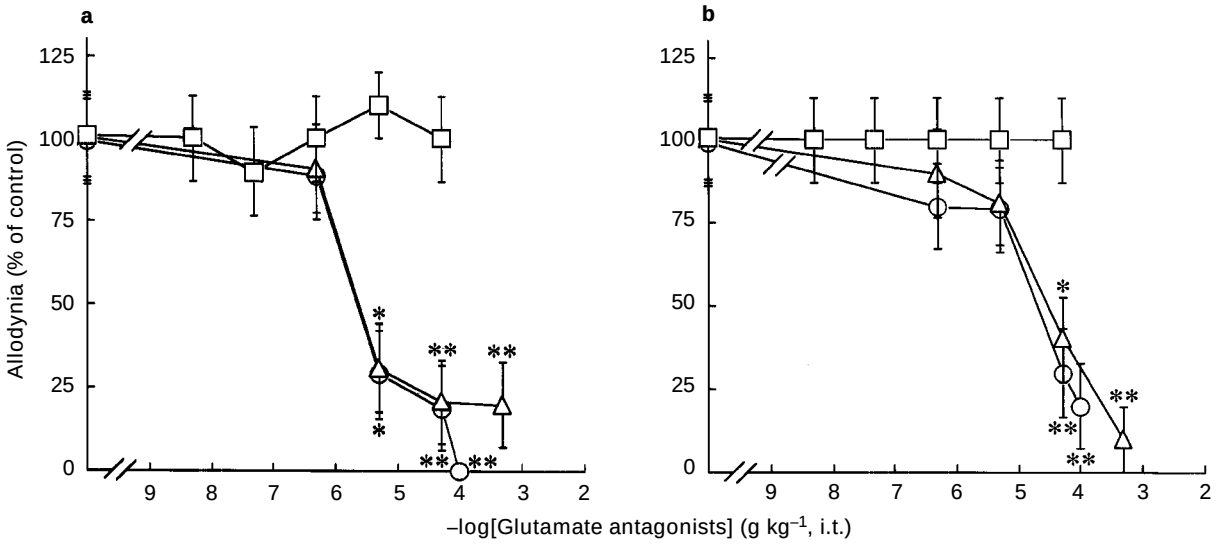

Figure 4 Effect of glutamate receptor antagonists on allodynia. Nociceptin (a, 2.5 ng kg^{-1}) or morphine (b, 0.5 mg kg^{-1}) was injected simultaneously with various doses of D-AP5 (○), GAMS (△) or L-AP4 (□) into the subarachnoid space. For assessment of allodynia see legend of Figure 3. The values shown are the mean and vertical lines s.e.mean ($n=6$). Statistical analyses were carried out by Williams' test. *$P<0.05$, **$P<0.01$, compared with nociceptin- or morphine-injected group.

receptors and that the $GABA_A$ antagonist bicuculline induced allodynia by activation of the mGluR and non-NMDA receptor (Onaka *et al*., 1996). Yaksh (1989) has suggested that low-threshold mechano-receptive primary afferents normally activate glycinergic and GABAergic inhibitory mechanisms and that these regulate the discharge evoked by the same afferents in wide dynamic range neurones in the deeper laminae of the dorsal horn, which are thought to be important in pain transmission. This inhibition may prevent innocuous cutaneous stimuli from being perceived as painful. The present study demonstrates that nociceptin induces allodynia through a mechanism similar to strychnine, consistent with the postulated mechanism of allodynia that glycinergic neurones in the dorsal horn are important in controlling the flow of information conveyed to the dorsal horn by myelinated low-threshold cutaneous afferents.

In the present study, we also characterized the morphine-induced allodynia evoked by innocuous tactile stimuli and initially demonstrated that glycine and taurine antagonized the morphine-induced allodynia (Figure 3b). Because brushing of the face or the forepaws did not give any response and the allodynia appeared limited to the caudal dermatomes, allodynia observed here may be caused by the action of morphine at the spinal level. The morphine-induced allodynia appeared to be mediated through the pathway common to the nociceptin-induced allodynia as: (1) the allodynia effects caused by morphine and nociceptin were dose-dependently blocked by glycine receptor agonists, but not by $GABA_A$ and $GABA_B$ receptor agonists; (2) these allodynia responses were attenuated by NMDA and non-NMDA receptor antagonists, but not by a mGluR antagonist; (3) these allodynia effects were attenuated by inhibitors of the NO-cyclicGMP system. However, (4) neither morphine- nor nociceptin-induced allodynia was blocked by the opioid receptor antagonist naloxone. Dose-dependency of the morphine-induced allodynia showed a bell-shaped pattern, similar to that of the nociceptin-induced re-

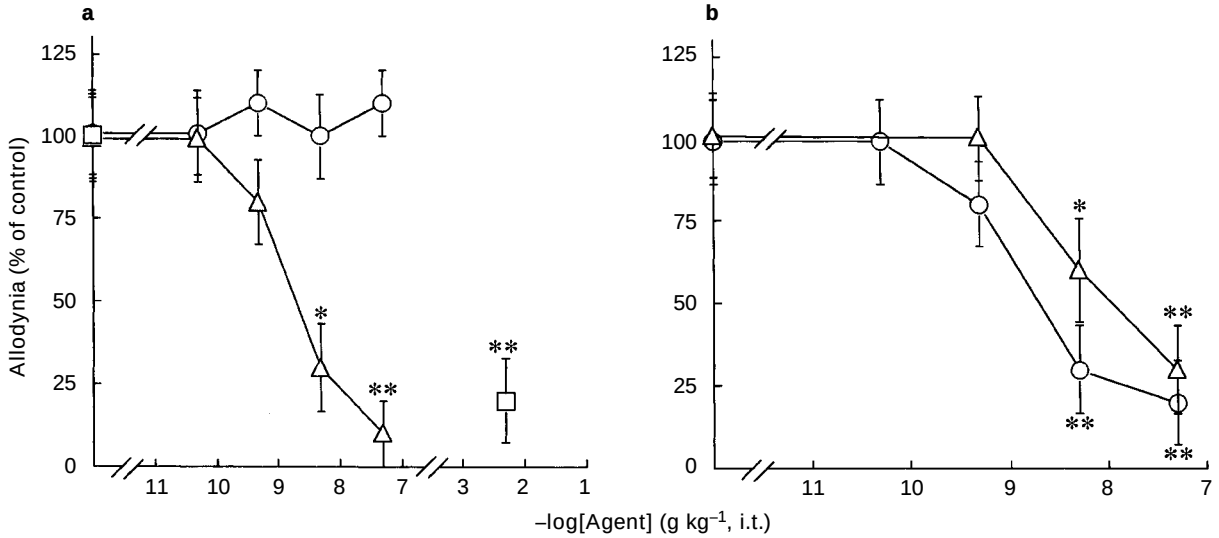

Figure 5 Effect of L-NAME, Hb or MB on allodynia. Nociceptin (a, 2.5 ng kg^{-1}) or morphine (b, 0.5 mg kg^{-1}) was injected simultaneously with various doses of L-NAME (○) or MB (△) into the subarachnoid space. Nociceptin (a, 2.5 ng kg^{-1}) was also injected simultaneously with 5 mg kg^{-1} Hb (□). For assessment of allodynia, see legend of Figure 3. The values shown are the mean and vertical lines s.e.mean ($n=6$). Statistical analyses were carried out by Williams' test. $^{*}P<0.05$, $^{**}P<0.01$, compared with nociceptin- or morphine-injected group.

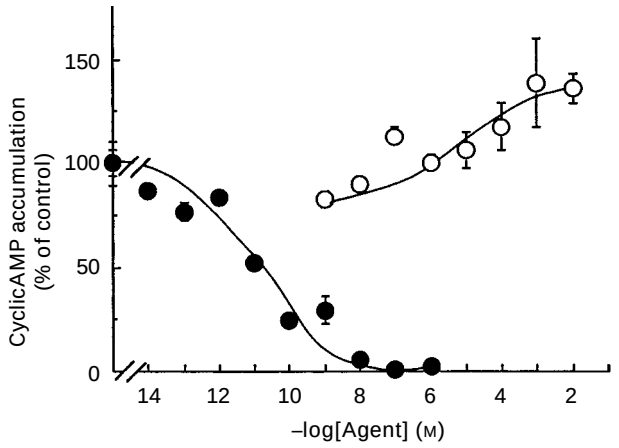

Figure 6 Effect of nociceptin and morphine on forskolin-induced cyclicAMP accumulation in CHO-ROR-C cells. Cells (2×10^5 cells/assay) were incubated with 3 μM forskolin in the presence of various concentrations of nociceptin (●) or morphine (○) and cyclicAMP accumulation was determined as described in Methods. The incubation buffer contained 0.5 mM 3-isobutyl-1-methylxanthine. The values shown are the mean and vertical lines s.e.mean ($n=3$).

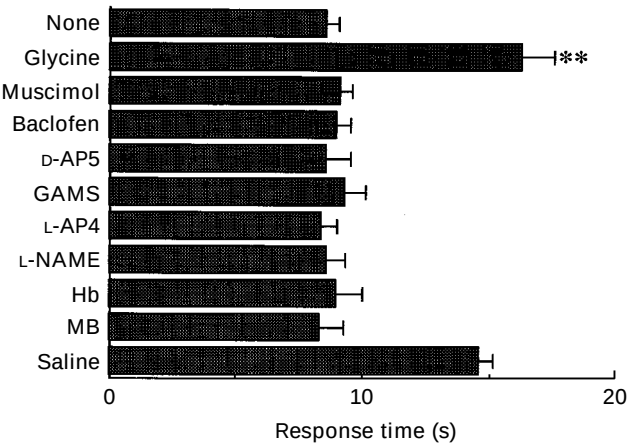

Figure 7 Effect of various agents on nociceptin-induced hyperalgesia. Nociceptin (50 pg kg^{-1}) was injected simultaneously with vehicle, glycine (50 μg kg^{-1}), muscimol (5.0 μg kg^{-1}), baclofen (0.5 μg kg^{-1}), D-AP5 (12.5 μg kg^{-1}), GAMS (50 μg kg^{-1}), L-AP4 (5.0 μg kg^{-1}), L-NAME (50 ng kg^{-1}), Hb (5.0 mg kg^{-1}) or MB (50 ng kg^{-1}) into the subarachnoid space. For assessment of hyperalgesia, see legend of Figure 2. The values shown are the mean ± s.e.mean ($n=10$). Statistical analyses were carried out by Duncan's test. $^{**}P<0.01$, compared with nociceptin-injected group (None, 7.9 ± 0.6 s, $n=10$).

sponse (Figure 2a), but morphine was five orders of magnitude less potent than nociceptin at inducing allodynia. Although nociceptin administered intracerebroventricularly was recently shown to reverse opioid-mediated (naloxone-sensitive) stress-induced antinociception and suggested to be functioning as an anti-opioid peptide (Mogil *et al*., 1996), i.t. nociceptin produced allodynia and hyperalgesia through naloxone-insensitive pathways. Because there was no significant difference in the latency period by the hot plate test between uninjected mice and i.t. saline mice (Figure 1c), it is likely that i.t. nociceptin increased pain sensation, rather than reversed stress-induced antinociception in our system. In view of the structural homology between the opioid and somatostatin receptors, which were proposed to derive from the same ancestor gene (Reisine & Bell, 1993), we examined possible interactions of morphine with nociceptin and somatostatin receptors for the induction of allodynia. In fact, morphine has recently been shown to cross-react with somatostatin receptors on T47D human breast cancer cells, and exert its antiproliferative action through the somatostatin receptor (Hatzoglou *et al*., 1995). However, the somatostatin antagonist did not block the morphine-induced allodynia. The results with CHO-ROR-C cells negated the possibility that high doses of morphine exerted their action through the nociceptin receptor (Figure 6). It was previously shown that the effect of high-dose morphine was not mediated by an interaction with postsynaptic receptors for amino acids including glutamate, glycine, and GABA (Bartlett *et al*., 1994). As yet, the type of receptor which interacts with morphine to produce allodynia remains unknown.

NO has been suggested to act as a neuronal messenger (Bredt & Snyder, 1992). Activation of the NMDA receptor results in the production of NO by NO synthase in a postsynaptic neurone from which it rapidly diffuses to enter the

presynaptic neurone. Thus, NO may modulate excitability and enhance the synaptic connection through activation of guanylate cyclase in presynaptic terminals and postsynaptic neurones. The nociceptin-induced allodynia was relieved by the NO scavenger Hb and MB, but not blocked by L-NAME (Figure 5a). Failure of L-NAME to prevent the nociceptin-induced allodynia suggests that NO may act as a neurotransmitter or a retrograde messenger, rather than act intracellularly in the neurone where it is produced. On the other hand, the morphine-induced allodynia was blocked by both L-NAME and MB (Figure 5b). Although the reason for the difference in the effects of L-NAME on allodynia evoked by nociceptin and morphine remains unknown, these results demonstrate that the nociceptin- and morphine-induced allodynia may be mediated by the glutamate receptor-NO system.

In *in situ* hybridization studies the cells that synthesize the nociceptin precursor mRNA were visualized in neurones of the tract of Lissauer, an intraspinal inhibitory pathway in the dorsal horn (Wall & Yaksh, 1978), and laminae I-III of dorsal horn of the spinal cord (Houtani *et al*., 1996; Okuda-Ashitaka *et al*., 1996). Glycine neurones are largely found in laminae II, III and the lateral aspect of laminae V, and receive a major monosynaptic input from myelinated low-threshold cutaneous primary afferents; glycine is considered to act as a postsynaptic inhibitory transmitter (Zarbin *et al*., 1981; Todd, 1990; Todd & Sullivan, 1990). L-Glutamate is known to be a neurotransmitter of primary afferents and descending projections from the upper brain and perhaps of some intrinsic spinal neurones (De Biasi & Rustioni, 1988; Headley & Grillner, 1990). Among the glutamate family, the NMDA and AMPA receptors have been shown to be located mainly at a postsynaptic site in the spinal cord, but the mGluR was likely to be located at a presynaptic site in the spinal cord (Headley & Grillner, 1990). The NMDA receptor channel is formed by the ε (NR2) and ξ (NR1) subfamilies (Nakanishi, 1992). The ξ1 (NR1) subunit of the NMDA receptor mRNA is found ubiquitously in the spinal cord and the ε1 subunit mRNA has been found in all regions of the gray matter, except for the lamina II, but the ε2 subunit mRNA has been shown to be restricted to the lamina II in mouse spinal cord (Watanabe *et al*., 1994). The NO synthase neurones and fibres have also been found to be located in superficial layers of the dorsal horn (Dun *et al*., 1992; Valtschanoff *et al*., 1992). These data support their involvement in mutual interactions in the transmission and modulation of pain. The perceptual processes seem to be complicated by the complex and efficacious neuronal circuits by which control afferent processing occurs and they appear to exert their effects through subtle changes in effect (Yaksh & Aimone, 1989). Because nociceptin clearly plays an important but complex role in both allodynia and hyperalgesia via different pain processing pathways, the continued pharmacological approach, along with the development of potent, competitive and selective nociceptin receptor antagonists, may provide us with an opportunity to learn more about the regulation of pain transmission under physiological and pathological conditions.

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