Loss of Antinociception Induced by Naloxone Benzoylhydrazone in Nociceptin Receptor-Knockout Mice*

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Nociceptin and nociceptin receptor, which show structural similarities to opioid peptides and opioid receptors, respectively, have been recently found to constitute a novel neuromodulatory system. In the brain, however, the physiological role of the modulation via the nociceptin receptor is still unclear. Administered nociceptin produces hyperalgesia and hypolocomotion, whereas the nociceptin receptor-knockout mice show no significant abnormalities in nociceptive thresholds and locomotion. To clarify possible involvement of the nociceptin receptor in the regulation of nociception and locomotion, we made use of the knockout mice and naloxone benzoylhydrazone (NalBzoH) identified originally as a ligand for opioid receptors. Experiments on the cultured cells transfected with the nociceptin receptor cDNA showed that NalBzoH competed with [3H]nociceptin binding and attenuated the nociceptin-induced inhibition of cAMP accumulation. Furthermore, behavioral studies demonstrated that NalBzoH completely inhibited nociceptin-induced hyperalgesia and hypolocomotion. It is therefore likely that NalBzoH can act as a potent antagonist for the nociceptin receptor in vivo. In wild-type mice, NalBzoH induced antinociception but did not affect locomotor activity. In contrast, in the knockout mice, no significant changes in nociception and locomotion were induced by NalBzoH. These results clearly suggest that the nociceptin system takes part in the physiological regulation of nociceptive thresholds but not in the basal modulation of locomotion.

Modulation by biologically active peptides is an essential feature in neurons. The opioid receptors exhibit a widespread distribution through the central and peripheral nervous systems and mediate physiological effects of endogenous opioid peptides and pharmacological actions of opioid analgesics. Recent DNA cloning studies have shown that the G protein-coupled opioid receptor family is comprised of three distinct opioid receptors (β-, µ- and κ-opioid receptors) and the nociceptin/orphanin FQ receptor (1–3). On the other hand, in previous pharmacological experiments, further refined classification of the opioid receptor subtypes has been suggested, for example the κ-receptor was classified into κ1-, κ2- and κ3- subtypes (4–6). The subclassified opioid receptor subtypes are still unknown at the molecular level, and the possibility that these are based on alternative splicing or derived from differences in post-translational modifications was proposed (1).

In contrast to the opioid peptides, nociceptin induces hyperalgesia and hypolocomotion by interacting specifically with the nociceptin receptors in the central nervous system (2, 3). To study the physiological roles of the nociceptin system, we have generated mutant mice lacking the nociceptin receptor. The knockout mice, lacking the responses of nociceptin-induced hyperalgesia and hypolocomotion, show no significant differences in nociceptive thresholds and locomotor activity to control mice (7). This suggests that the nociceptin receptor plays no essential role in regulating either nociceptive sensitivity or locomotor activity. However, these experimental results cannot eliminate the possibility that the nociceptin receptor is involved in the modulation of both the mechanisms on the basal level because the redundancy of other regulatory systems may compensate for the abnormalities caused by the deficiency of the nociceptin receptor. On the other hand, the loss of the nociceptin receptor results in abnormal hearing ability, demonstrating that the nociceptin system is essential for the regulation of the auditory system physiologically (7). Although many pharmacological actions have been suggested so far based on effects of nociceptin administration, the physiological roles of the nociceptin receptor have not yet been fully elucidated (8). One major reason for this is that specific non-peptide drugs modulating the nociceptin receptor activity in vivo are not available.

Naloxone benzoylhydrazone (NalBzoH)1 is a derivative compound of the µ-opioid receptor antagonist, naloxone, and produces antinociceptive effects in vivo (9). Previous ligand-binding studies suggested that NalBzoH interacts with the µ-, κ1-, and κ3-opioid receptor subtypes (4, 5). Because the κ-opioid receptor defined by cloning studies shows the pharmacological characteristics of the κ1-subtype when the cDNA is functionally expressed in cultured cells, the molecular profile of the κ3-subtype has yet to be elucidated. Recent studies have suggested a close relationship between the pharmacological κ3-subtype and the molecular biological nociceptin receptor, but some data have negated the relationship (10, 11). Thus, the pharmacological characteristics of NalBzoH remained to be investigated.

From the results of ligand-binding experiments using the nociceptin receptor expressed from the cDNA and behavioral studies contrasting drug-induced responses between the nociceptin-deficient and wild-type mice, we report here the identi-

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1 The abbreviations used are: NalBzoH, naloxone benzoylhydrazone; CHO, Chinese hamster ovary.
fication of NalBzoH as an antagonist for the nociceptin receptor and the contribution of the nociceptin system to the regulation of the physiological nociceptive sensitivity.

**EXPERIMENTAL PROCEDURES**

**Ligand-binding Assay**—Chinese hamster ovary (CHO) cells expressing the rat nociceptin receptor (ROR-C) were established as described previously (12, 13). Cells were washed with phosphate-buffered saline and homogenized in 50 mM Tris-HCl, pH 7.5, and 5 mM MgCl₂. The homogenate was centrifuged at 1,000 g for 5 min, the supernatant was centrifuged at 15,000 g for 30 min, and the resulting pellet was suspended in the same buffer and used for the binding assay. The binding reaction was performed at room temperature for 60 min with the membrane preparations (35–64 μg) in a solution (0.2 ml) containing 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, and various concentrations of [3H]nociceptin (45 Ci/mmol; NEN Life Science Products). After incubation, samples were collected on a GF/C filter (Whatman), washed with 15 ml of the buffer solution, and then counted for radioactivity. Non-specific binding was measured in the presence of an excess amount of unlabeled nociceptin. The dissociation constant (Kd) for [3H]nociceptin was determined by Scatchard analysis, and apparent Kd values for unlabeled ligands were obtained by measuring displacement of [3H]nociceptin (3 nM) according the equation Kd = IC₅₀/(1 + S) where S = (concentration of [3H]nociceptin)/(Kd of [3H]nociceptin).

**Cyclic AMP Assay**—The cyclic AMP assay was carried out essentially as described previously (13). CHO cells expressing the nociceptin receptor were cultured in 24-well plates (2.5 x 10⁵ cells/well) and preincubated for 5 min with 0.72 ml of Krebs-Henseleit buffer (111 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃, 11 mM glucose, pH 7.4) supplemented with 0.5 mM 3-isobutyl-1-methylxanthine. Reactions were started by addition of 0.08 ml of 100 mM forskolin-containing test agents. After 10 min, the incubation reactions were terminated by addition of ice-cold 1 M perchloric acid solution (0.2 ml), and cell debris was removed by centrifugation at 15,000 x g for 10 min. The supernatants were neutralized with 2 ml of KHCO₃ (0.08 ml) and centrifuged at 15,000 x g for 10 min. The resulting supernatants were subjected to enzyme immunoassays (cAMP enzyme immunoassay system; Pharmacia Amersham Corp.).

**Antinociceptive Tests and Measurement of Locomotor Activity**—We used the male mice lacking the nociceptin receptor and wild-type mice (9–12 weeks old) as reported by Nishi et al. (7).

In the tail-flick test, the latency to withdraw the tail from a focused light stimulus was measured electronically, using a photocell. The intensity of the heat stimuli was set as inducing approximately 10-s base-line latencies in wild-type mice. A maximal latency of 20 s was assigned to minimize tissue damage. The base-line latencies were determined at least 1 h before the drug treatment for all animals as the mean of two trials. Tail-flick latencies were measured at 10, 15, 60, 5, 10, 15, 60, and 5, respectively, before the observation. The number of writhes, characterized by a wave of contraction of the abdominal muscles followed by extension of the hind limbs, was counted for 10 min, beginning 5 min after acetamid injection.

In the locomotion test, each animal was placed in a transparent acrylic cage immediately after the tail-flick test, and locomotor activity was measured for 30 min using digital counters with infrared sensors (Scanet SV-10; Toyo Sangyo, Toyama, Japan).
RESULTS AND DISCUSSION

**Naloxone Benzoylhydrazone as Nociceptin Receptor Antagonist**—First, we examined the ligand-binding properties of the nociceptin receptor using membrane preparations from the CHO clone transfected with the rat nociceptin receptor (ROR-C) cDNA (12, 13). Saturation analysis of \[^{3}H\]nociceptin binding revealed that the ROR-C proteins expressed in the cells are capable of binding \[^{3}H\]nociceptin, and Scatchard analysis yielded a \(K_d\) of 0.54 nM (Fig. 1A). Displacement tests were performed to examine the effects of NalBzoH on the nociceptin receptor. The results in Fig. 1B demonstrate that NalBzoH competed with nociceptin binding effectively and the apparent \(K_d\) of NalBzoH was calculated to be \(25 \text{ nM}\). The results in Fig. 1Bdemonstrate that nociceptin and Nal-BzoH interact with the expressed nociceptin receptor at the same site. This competitive binding is also supported by a recent report that NalBzoH inhibited \[^{[Tyr14]}\]nociceptin binding to microsomes prepared from the brain (14). These facts suggest that NalBzoH should act as an agonist or antagonist at the nociceptin receptor. As we reported previously, in contrast to nociceptin, NalBzoH (10 pm to 1 \(\mu\)M) does not inhibit forskolin-induced cAMP accumulation in CHO cells. Therefore it seems appropriate to conclude that NalBzoH acts as a high affinity antagonist for the nociceptin receptor. This conclusion was further supported by the experimental data (Fig. 2), in which NalBzoH blocked the nociceptin-induced inhibition of cAMP accumulation in CHO cells expressing the nociceptin receptor. The \(K_d\) value of NalBzoH in this functional assay was severalfold higher than that calculated in the binding assay. Although the reason for this is unclear, a similar discrepancy of \(K_d\) values has been observed in the case of nociceptin as an agonist ligand (2, 3, 13).

**Nociception and Nociceptin Receptor**—To compare nociceptive responses induced by various drugs between the nociceptin receptor-deficient and wild-type mice, we conducted the heat tail-flick test. No measurable changes were detected in the basal nociceptive thresholds between the genotypes in this test as described previously (7, 15). As expected, morphine (\(\mu\)-opioid receptor agonist) and U-50,488H (\(\kappa_1\)-opioid receptor agonist) induced antinociceptive effects dose-dependently in wild-type mice (Fig. 3A). These effects were also seen in the mutant mice, and both drugs showed similar effective doses in the mutant and wild-type animals. As previously reported (5, 7), NalBzoH and nociceptin induced antinociceptive and hyperalgesic effects dose-dependently in wild-type mice (Fig. 3A). These effects were also seen in the mutant mice, and both drugs showed similar effective doses in the mutant and wild-type animals. As previously reported (5, 7), NalBzoH and nociceptin induced antinociceptive and hyperalgesic effects, respectively, in wild-type mice. However, the knockout mice lacked both the effects induced by NalBzoH and nociceptin. Similar results were obtained in the acetic acid-induced writhing test, a method allowing detection of relatively smaller changes in nociceptive thresholds compared with the tail-flick test (Fig. 4). Furthermore, NalBzoH effectively inhibited hy-
peralgesia induced by nociceptin in wild-type mice (Fig. 3B). However, the hyperalgesia was affected by neither U-50,488H at the dose inducing analgesia in normal conditions nor 5 mg/kg of naloxone which antagonizes the action of morphine. Furthermore, the antagonistic effect of NalBzoH on the nociceptin-induced hyperalgesia was not affected by naloxone. The results clearly show that the knockout mice lack the signal transduction system mediating both nociceptin-induced hyperalgesia and NalBzoH-induced antinociception. Thereby the nociceptin receptor is most likely to correspond to the primary reacting site of NalBzoH-induced antinociception in wild-type mice. Because we observed no significant NalBzoH-induced effect on nociceptive thresholds in the knockout mice retaining sensitivities to \( \mu \) - and \( \kappa \)-opioid receptor agonists, it seems that NalBzoH at 75 mg/kg in both the tail-flick and writhing tests did not affect \( \mu \)- and \( \kappa \)-opioid receptors. Thus, the specific inhibitory effect of NalBzoH on nociceptin-induced hyperalgesia strongly indicates that NalBzoH acts as a potent antagonist for the nociceptin receptor in vivo. Moreover, it is reasonable to assume that the NalBzoH-induced antinociception in wild-type mice is due to disregulation of nociceptive thresholds by the inhibition of the nociceptin receptor. Our findings suggest that the nociceptin receptor participates in the physiological regulation of nociceptive thresholds, and also that in the knockout mice neuroregulatory systems other than the nociceptin system compensate for abnormalities in nociceptive thresholds caused by the deficiency of the nociceptin receptor. This should stimulate further investigations to locate regions responsible for the nociceptive control exerted by the nociceptin receptor. We now know the distribution patterns of both nociceptin precursor and nociceptin receptor in the central nervous system (8). Areas relating to perception and modulation of pain include the periaqueductal gray and cerebral cortex, where expression of both nociceptin precursor and nociceptin receptor is found. These regions may be the best candidate sites responsible for the modulation of nociceptive sensitivity.

Locomotion and Nociceptin Receptor—Next, we examined whether the nociceptin system is involved in locomotor activity. As described previously (7), the spontaneous locomotion in the nociceptin receptor-knockout mice was slightly lower than that in wild-type mice, and nociceptin induced hypolocomotion in wild-type mice while the knockout mice lacked the effect (Fig. 5A).

**Fig. 4.** Antinociceptive effects of opioid-related drugs in the acetic acid induced-writhing assay. The test protocol is described in the text. The results are expressed as the mean ± S.E. of the writhing counts for each mouse (n = 20–25 in each group). **p < 0.05 versus corresponding pre-value (Dunnett multiple comparisons test).

**Fig. 5.** Locomotor activity. A, effects of nociceptin and NalBzoH on the locomotor activity in the nociceptin receptor-knockout (\( -/- \)) and wild-type (+/+ ) mice. The test protocol is described in the text. Results were analyzed as total counts for 30 min and are given as the mean ± S.E. (n = 6–10 in each group). **p < 0.05 versus control (cont.) group (Dunnett multiple comparisons test). B, effect of NalBzoH on nociceptin-induced hypolocomotion in wild-type mice. Results were analyzed as total counts for 30 min and are given as the mean ± S.E. (n = 6–10 in each group). **p < 0.05 versus control (cont.) group. ††p < 0.05 versus (nociceptin + vehicle (V))-treated group (Dunnett multiple comparisons test).
Administration of NalBzoH resulted in no significant changes of locomotion in either mouse genotype (Fig. 5A). In wild-type mice, however, nociceptin-induced hypolocomotion was efficiently inhibited by NalBzoH at the dose inducing the antinociceptive effect. In contrast, U-50,488H at the effective dose for antinociception barely attenuated the hypoactivity (Fig. 3B).

The inhibitory effect of NalBzoH on nociceptin-induced hypolocomotion is probably due to an antagonistic action at the nociceptin receptor as discussed above. If the nociceptin receptor contributes to the regulation of locomotor activity, NalBzoH should induce hyperactivity in wild-type mice. However, we could not find any significant changes in locomotor activity when we administered NalBzoH in wild-type mice. This result strongly suggests that the nociceptin receptor does not participate in the control of spontaneous locomotion physiologically. Perhaps nociceptin-induced hypolocomotion is an adverse effect caused by nociceptin-induced hyperalgesia. Behavioral studies reported recently showed disruption of balance following intracerebroventricular administration of nociceptin in rats (16). Since our results here did not deal with motor coordination, the possible involvement of the nociceptin receptor in the regulation remains to be investigated.

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