Profiles of the Antinociceptive Effect of R-84760, a Selective \(\kappa\)-Opioid Receptor Agonist, in the Formalin Test in Mice

Kenji Fujibayashi and Yoshio Iizuka

Biological Research Laboratories, Sankyo Co., Ltd., 2-58, 1-chome, Hiromachi, Shinagawa-ku, Tokyo 140, Japan

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ABSTRACT—The antinociceptive effect of a selective \(\kappa\)-opioid receptor agonist R-84760, (3R)-3-(1-pyrrolidinylmethyl)-4-[(1S)-5,6-dichloro-1-indancarbonyl]-tetrahydro-1,4-thiazine hydrochloride, in the second phase of the formalin test, a model of tonic pain, was examined in mice. R-84760 had a 2700 times more potent antinociceptive effect than morphine. The effect of R-84760 was antagonized by subcutaneously administered nor-binaltorphimine, a \(\kappa\)-selective opioid receptor antagonist. Both intracerebroventricularly and intrathecally administered nor-binaltorphimine partially antagonized the antinociceptive effect of R-84760. Intrathecally administered phentolamine, an \(\alpha\)-adrenoceptor antagonist, attenuated and desipramine, a noradrenaline reuptake inhibitor, augmented the antinociceptive effect of R-84760. Intrathecally administered noradrenaline reuptake inhibitor, augmented the antinociceptive effect of R-84760. Intrathecally administered noradrenaline attenuated the nociceptive response in the second phase of the formalin test. Intrathecally administered (\(\pm\))-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), an N-methyl-D-aspartate (NMDA)-receptor antagonist, reduced and threo-\(\beta\)-hydroxyaspartate, a reuptake inhibitor of glutamate, augmented the second phase nociceptive response. However, R-84760 did not influence the intrathecally injected NMDA-induced nociceptive response. These results suggest the following: R-84760 produces an extremely potent antinociceptive effect against tonic pain through the \(\kappa\)-opioid receptors; the sites of action of subcutaneously administered R-84760 are the supraspinal and spinal loci in the central nervous system; and a part of the mechanism of the antinociceptive effect of R-84760 is activation of the descending noradrenergic pathway.

Keywords: R-84760, \(\kappa\)-Opioid receptor agonist, Formalin test, Tonic pain, Antinociceptive effect

The compound R-84760 was reported to be a novel \(\kappa\)-opioid receptor agonist (1), of which the chemical structure is (3R)-3-(1-pyrrolidinylmethyl)-4-[(1S)-5,6-dichloro-1-indancarbonyl]-tetrahydro-1,4-thiazine hydrochloride. R-84760 has a higher affinity for the \(\kappa\)-opioid receptor than CI-977 (1), which is also a potent \(\kappa\)-opioid receptor agonist (2). The selectivity of R-84760 for the \(\kappa\)-opioid receptor is higher than that of U-69593 (1), a compound with high selectivity for the \(\kappa\)-opioid receptor (2, 3). \(\kappa\)-Opioid receptor agonists have been shown to have antinociceptive activity, and R-84760, in fact, has antinociceptive activity in the phenylquinone writhing test in mice. Its potency is 5.3, 360 and 370 times higher than CI-977, U-50488 (a \(\kappa\)-selective opioid receptor agonist) and morphine, respectively, by subcutaneous (s.c.) administration (1).

It has been suggested that tonic nociception in experimental models is more similar to clinical pain than phasic nociception models such as the tail-flick and hot-plate tests (4, 5). As one of the tonic nociception models, the formalin test (6–8) has been widely employed. Especially, the second phase response in this test has been used in recent studies (9) including those dealing with the antinociceptive effect of \(\kappa\)-opioid receptor agonists (10–14). In the present experiments, the antinociceptive activity of R-84760 was assessed in the second phase of formalin tests in mice. The site of action and possible involvement of the nervous systems in R-84760 action were also examined.

MATERIALS AND METHODS

Animals

Male ddY mice weighing 17–27 g were obtained from Japan SLC, Inc. (Shizuoka). Mice were housed for at least three days in an animal room with controlled temperature (23 ± 1°C), humidity (55 ± 5%) and artificial lighting (07:00–19:00). Food and water were available ad libitum.
Formalin test

Each mouse was placed and left for 30 min in a transparent plastic cylinder (inner diameter, 24 cm; height, 19 cm) so that it would become accustomed to the experimental environment. The mice were s.c. injected with 20 µl of 3.5% formalin to the planter region of the right hind paw after acclimation. The total duration of licking of the formalin-injected paw was recorded between 15 and 35 min after the formalin injection. This observation period is included in the period of the second phase response (Fig. 1). A mirror was set across the plastic cylinder in order to observe the response precisely when the backs of the mice were to the observer.

R-84760, morphine and naloxone were s.c. injected 5, 35 and 10 min, respectively, before starting the observation periods. Nor-binaltorphimine (nor-BNI) was s.c. injected 24 hr before the tests according to a previous report (15). Intracerebroventricular (i.c.v.) (16) and intrathecal (i.t.) (17) injections of the drugs in a volume of 5 µl were performed 10 min before the start of observation, except for noradrenaline (NA) which was administered 2 min before observation. I.c.v. injections were performed under light ether anesthesia.

When the antinociceptive effect of R-84760 on the first phase response was evaluated, the drug was administered 13 min before the formalin injection and the duration of licking was recorded between 0 and 10 min after the formalin injection.

N-methyl-D-aspartate (NMDA)-induced nociceptive responses

Each mouse was placed in a transparent plastic cage (24 x 14 x 12 cm). After acclimation for 30 min, 0.32 nmol/mouse NMDA was injected i.t. Licking towards the caudal part of the body was elicited with the minor incidence of hind limb scratching immediately after the NMDA injection. In the present experiment, only licking was taken as a nociceptive response, and the total duration of the response during the first 2 min after injection of NMDA was recorded. (+)-3-(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) was i.t. and R-84760 was s.c. administered 20 min and 15 min before the NMDA injection, respectively.

Drugs

R-84760 and nor-BNI dihydrochloride were synthesized in the Medicinal Chemistry Research Laboratories, Sankyo Co., Ltd. (Tokyo). Morphine hydrochloride, naloxone hydrochloride (Sankyo Co., Ltd., Tokyo); DL-threo-β-hydroxyaspartate, NMDA, phentolamine hydrochloride, desipramine hydrochloride, NA bitartrate and bicuculline methiodide (Sigma Chemical Co., St. Louis, MO, USA); CPP and phaclofen (Research Biochemicals, Inc., Natick, MA, USA) were purchased. Methysergide bimaleate was generously supplied by Sandoz, Ltd. (Basel, Switzerland). All the drugs were dissolved in saline.

Statistical analyses

Comparisons were made by the Mann-Whitney test for independent samples. Multiple comparisons against the control were made by the Kruskal-Wallis test followed by Dunnett’s test. Antinociceptive potency was expressed as an ID$_{50}$, the dose at which the duration of the nociceptive responses was reduced to 50% of the mean control value. ID$_{50}$ values were determined by linear regression analysis from the percentage inhibition values.

RESULTS

Antinociceptive effect of subcutaneously injected R-84760

The s.c. injection of R-84760 dose-dependently reduced the duration of licking in both the first and second phases of the formalin test with ID$_{50}$ values (confidence limits in parentheses) of 2.4 (2.0–2.8) and 0.97 (0.67–1.38) µg/kg, respectively. ID$_{50}$ values for U-50488 and morphine for the second phase responses were 0.15 (0.08–0.30) and 2.6 (1.9–3.5) mg/kg, respectively. Dose-response curves of the effect on the second phase response are shown in Fig. 2. The antinociceptive effect of R-84760 in the second phase is 155 and 2700 times more potent than those of U-50488 and morphine, respectively, based on the ID$_{50}$ values.
Effect of opioid receptor antagonists

The s.c. injection of naloxone, a relatively μ-selective opioid receptor antagonist, significantly antagonized the antinociceptive effect of morphine (3.5 mg/kg, s.c.) at doses of 32 and 100 μg/kg in the second phase of the formalin test (Fig. 3A). On the other hand, naloxone could not significantly antagonize the effect of R-84760 (2.5 μg/kg, s.c.) even at a dose of 100 μg/kg (Fig. 3A). Naloxone, however, antagonized the effect of R-84760 at a dose as high as 1 mg/kg (data not shown). The doses of R-84760 and morphine used were submaximal. Naloxone (1 mg/kg, s.c.) alone did not influence the licking response.

The s.c. injection of nor-binaltorphimine (nor-BNI), a κ-selective opioid receptor antagonist, significantly antagonized the antinociceptive effect of R-84760 (2.5 μg/kg, s.c.) at doses of 10 and 32 mg/kg in the second phase of the formalin test (Fig. 3B). On the other hand, nor-BNI could not significantly antagonize the effect of morphine (3.5 mg/kg, s.c.) even at a dose of 32 mg/kg (Fig. 3B). Nor-BNI (32 mg/kg, s.c.) alone did not influence the licking responses.

The i.c.v. injection of nor-BNI attenuated the R-84760-induced antinociception (Fig. 4A). By i.t. injection, nor-BNI was also effective (Fig. 4B). Centrally administered nor-BNI alone did not significantly influence the licking response (Fig. 4, A and B).

The mechanism of the antinociceptive effect of R-84760 and neuronal mechanisms

The i.t. injection of phentolamine, an α-adrenoceptor antagonist, at 1 nmol/mouse significantly reduced the antinociceptive effect induced by R-84760 (Fig. 5A). In contrast, i.t. injected desipramine (1 nmol/mouse), a NA re-uptake inhibitor, significantly potentiated the effect of R-84760 (Fig. 5B). Phentolamine or desipramine alone did not influence the licking response (Fig. 5, A and B). NA, injected i.t., attenuated the licking response of mice in the formalin test dose dependently (Fig. 5C).

The i.t. injection of methysergide (0.1 nmol/mouse), a serotonin (5-HT)-receptor antagonist, did not recover the reduced licking response by R-84760. (Fig. 6A). At a dose of 1 nmol/mouse, methysergide itself reduced the licking response of the control mice and did not influence the antinociceptive effect of R-84760 (Fig. 6A).

The i.t. injection of bicuculline methiodide (0.1 nmol/mouse), a γ-aminobutyric acid (GABA)A-receptor antagonist, and phaclofen (10 nmol/mouse), a GABAB-receptor antagonist, did not influence the reduced licking...
Fig. 4. Effect of i.c.v. (A) and i.t. (B) injected nor-BNI (nmol/mouse) on the antinociceptive effect of R-84760 (2.5 μg/kg, s.c.) in the second phase of the formalin test in mice. Data are shown as mean values of the total duration of licking with S.E. The numbers of animals used are shown in parentheses. *P<0.05, **P<0.01, compared with the respective saline+R-84760-treated groups.

Fig. 5. Effects of phentolamine (PHEN, nmol/mouse, i.t.; A) and desipramine (DESI, nmol/mouse, i.t.; B) on the antinociceptive effect of R-84760 (R-84; 1.3 μg/kg, s.c., A; 0.63 μg/kg, s.c., B) and the antinociceptive effect of noradrenaline (NA, nmol/mouse, i.t.; C), in the second phase of the formalin test in mice. Data are shown as mean values of the total duration of licking with S.E. The numbers of animals used are shown in parentheses. *P<0.05, compared with the respective saline+R-84760-treated groups (A and B) and with the saline-treated group (C).

Fig. 6. Absence of the effect of methysergide (METH, nmol/mouse, i.t.; A), bicuculline methiodide (BICU, nmol/mouse, i.t.; B) and phaclofen (PHAC, nmol/mouse, i.t.; C) on the antinociceptive effect of R-84760 (R-84; 1.3 μg/kg, s.c.) in the second phase of the formalin test in mice. Data are shown as mean values of the total duration of licking with S.E. The numbers of animals used are shown in parentheses. *P<0.05, compared with the saline + saline-treated group.
response by R-84760 (Fig. 6, B and C).

CPP, a competitive NMDA-receptor antagonist, reduced the total duration of the licking response of the second phase dose-dependently (Fig. 7A). CPP was i.t. injected 5 min after the injection of formalin, the time when the first phase response had almost passed (Fig. 1). In contrast, the licking response was significantly augmented by i.t. administered dl-threo-β-hydroxyaspartate, a reuptake inhibitor of glutamate, at a dose of 10 nmol/mouse (Fig. 7B).

NMDA induced nociceptive responses such as licking towards the caudal part of the body following i.t. injection. I.t. administered CPP almost abolished this response at a dose of 0.1 nmol/mouse (data not shown). The licking response induced by i.t. injected NMDA was not affected by R-84760 (Fig. 8).

DISCUSSION

R-84760 produced a highly potent antinociceptive effect against both first (phasic) and second (tonic) phase nociception. The present experiments were performed mainly on second phase nociception which is considered to have relevance to clinical pain. The potency ratio between R-84760 and morphine (R-84760/morphine) of 2700 in the second phase of the formalin test is higher than that of 370 in the phenylquinone writhing test (1). Similarly, the potency ratio between U-50488 and morphine (U-50488/morphine) of 17 obtained in the formalin test was larger than that in the phenylquinone writhing test where U-50488 and morphine were almost equipotent (1). These results indicate that κ-opioid receptor agonists are more effective than morphine in the formalin test.

The antinociceptive effect of R-84760 was less sensitive to the antagonism by naloxone than the effect of morphine. On the other hand, R-84760 was antagonized by nor-BNI (18) more sensitively than the effect of morphine. These results indicate that the antinociceptive effect of R-84760 in the formalin test is mediated through κ-opioid receptors, as the effect in the phenylquinone writhing test (1). The possibility of a δ-opioid receptor-mediated effect of R-84760 is excluded by the extremely low affinity of R-84760 for the δ-opioid receptor (1).

Partial antagonism of s.c. administered R-84760 by i.c.v. and i.t. injected nor-BNI indicates that both the supraspinal and spinal loci of the central nervous system are the action sites of R-84760 for its antinociceptive effect in the formalin test. This finding is consistent with a previous report that the potencies of i.c.v. and i.t. injected U-50488 were similar in inhibiting formalin-induced licking in mice (14). Almost the same results were obtained with E-2078, a peptide κ-opioid receptor agonist (19). On the other hand, however, different results were obtained in the tail-flick and hot-plate tests, both thermal pain models. Here, it was shown that U-50488 (20, 21) and U-62066 (spiradoline, a κ-opioid receptor agonist; refs. 21, 22) mainly and E-2078 (19) preferentially act in the spinal cord. In addition, the main site of action of U-50488 was shown to be the spinal cord in the acetic acid writhing test (23) which is a chemical pain model, as is the formalin test. Therefore, the differences in the action sites of κ-opioid receptor agonists between the formalin test and other tests may be a result of their differences in mode or duration of nociceptive stimuli.
Endogenous monoamines, such as NA and 5-HT, were suggested to contribute to the antinociception induced by opioids (24), including \(\kappa\)-opioid receptor agonists (22, 25–28). Involvement of the mechanisms was assessed in the action of R-84760 in the formalin test. There are conflicting reports concerning the involvement of descending noradrenaline-containing neurons in the antinociceptive mechanism of \(\kappa\)-opioid receptor agonists in the tail-flick test in mice. Nakazawa et al. (27) demonstrated lowered antinociceptive effects of \(\kappa\)-opioid receptor agonists in mice treated with toxins targeted to the noradrenaline-containing neurons. On the other hand, Ho and Takemori (26) showed that the antinociceptive potency of a \(\kappa\)-opioid receptor agonist was not altered by \(\alpha\)-adrenoceptor antagonists treated s.c. In the present experiments, i.t. administered phenolamine and desipramine, respectively, reduced and potentiated the antinociceptive effect of R-84760. This suggests that activation of noradrenaline-containing neurons in the spinal cord is at least partly involved in the antinociceptive effect of R-84760 in the second phase of the formalin test in mice. R-84760 may activate descending noradrenaline-containing neurons; and, as a result, the excess NA released from the nerve terminals in the spinal cord plays a role in producing the antinociceptive effect, through the \(\alpha\)-adrenoceptors. The antinociceptive effect of i.t. injected NA in the formalin test supports this possibility.

Several reports have shown the involvement of serotonergic mechanisms in the antinociceptive action of \(\kappa\)-opioid receptor agonists in thermal pain models (22, 25–27). One of these reports showed that i.t. injected methysergide reduced the antinociceptive potencies of both i.c.v. and i.t. injected U-50488 in the tail-flick test in mice (26). In the present experiments, however, i.t. methysergide failed to attenuate the antinociceptive effect of R-84760, indicating that activation of methysergide-sensitive 5-HT receptor subtypes (5-HT\(_{2A}\) and 5-HT\(_{2C}\), refs. 29, 30) in the spinal cord are not involved in the antinociceptive mechanisms of R-84760 in the second phase of the formalin test. This discrepancy in the effect of methysergide may be due to differences in the mode or duration of nociceptive stimuli.

The participation of GABA systems in the antinociception induced by \(\mu\)-opioid receptor agonists, such as that by morphine, is somewhat controversial (24, 31). In addition, few experiments have been reported so far on the involvement of the GABA systems, especially those in the spinal cord, in the antinociceptive effect of \(\kappa\)-opioid receptor agonists. In the present experiments, bicuculline methiodide and phaclofen, administered i.t., were ineffective on the effect of R-84760. The doses of bicuculline methiodide and phaclofen were sufficient to antagonize the action of endogenous GABA (32). Therefore, it is considered that the GABA\(_A\) and GABA\(_B\) receptors in the spinal cord are not involved in the antinociceptive mechanisms of R-84760.

The mechanism of action of opioids at the spinal level has been postulated to be both presynaptic and postsynaptic (24). This possibility was examined in the action of R-84760, paying attention to glutamate as one of the nociceptive transmitters in the spinal cord. The i.t. injection of a NMDA-receptor antagonist (CPP) and glutamate reuptake inhibitor (\(\text{dL-\text{threo-}}\beta\)-hydroxysaspartate), respectively, reduced and augmented the licking response in the second phase of the formalin test. These results suggest that endogenous glutamate, released from the nerve terminal, plays a role in the nociception of the second phase of the formalin test. The failure of R-84760 to inhibit the intrathecal NMDA-induced licking response shows that R-84760 does not inhibit glutamate-mediated nociceptive transmission postsynaptically. R-84760 may inhibit the release of glutamate in the spinal cord.

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