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European Journal of Pharmacology 343 (1998) 171–177

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# Effects of R-84760, a selective κ-opioid receptor agonist, on nociceptive reflex in isolated neonatal rat spinal cord

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Received 10 April 1997; revised 24 November 1997; accepted 25 November 1997

**Abstract**

We tested the effects of (3*R*)-3-(1-pyrrolidinylmethyl)-4-[(1*S*)-5,6-dichloro-1-indancarbonyl]-2,3,5,6-tetrahydro-1,4-thiazine hydrochloride (R-84760), a selective κ-opioid receptor agonist, on the slow ventral root potential in the isolated spinal cord of neonatal rats. R-84760 at 10 nM decreased the slow ventral root potential to 35% of the control, leaving the monosynaptic reflex unaffected. The depressant effect of R-84760 progressed slowly for 60 min to the maximum and recovered slightly after removal of the drug from the perfusing solution. This contrasts with [D-Ala$^2$, MePhe$^4$, Gly-ol$^5$]enkephalin (DAMGO) or [MeTyr$^1$, MeArg$^7$, D-Leu-NHEt$^8$]dynorphin A-(1–8) (E-2078) which attained their maximum depressant effect within 15 min with recovery immediately after washout. Reversibility of the R-84760 effect was observed in vivo in antinociceptive tests in mice. R-84760 reduced the depolarization induced by substance P or L-glutamate in the normal solution, but not in the presence of tetrodotoxin at 0.3 μM. Naloxone inhibited the effect of R-84760 at a higher concentration (1 μM) than that (0.1 μM) needed to antagonize the effect of DAMGO. In contrast, R-84760 was more sensitive to nor-binaltorphimine than was DAMGO. The results show that R-84760 selectively inhibits the nociceptive response presynaptically through κ-opioid receptors and that the inhibitory effect is characteristic, with long duration, in the neonatal rat spinal cord. © 1998 Elsevier Science B.V.

*Keywords:* κ-Opioid receptor agonist; R-84760; DAMGO ([D-Ala$^2$, MePhe$^4$, Gly-ol$^5$]enkephalin); E-2078; Nociceptive reflex; Spinal cord, neonatal rat

## 1. Introduction

(3*R*)-3-(1-pyrrolidinylmethyl)-4-[(1*S*)-5,6-dichloro-1-indancarbonyl]-2,3,5,6-tetrahydro-1,4-thiazine hydrochloride (R-84760) has been shown to be a selective and potent κ-opioid receptor agonist (Fujibayashi et al., 1994). R-84760 displays an antinociceptive effect in the phenylquinone writhing test in mice (Fujibayashi et al., 1994) and formalin test in mice (Fujibayashi and Iizuka, 1995) and rats (Fujibayashi et al., 1996) with potencies 240–2600 times higher than that of morphine on subcutaneous ($ED_{50}$; 0.97–1.4 μg/kg) or oral administration ($ED_{50}$; 13 μg/kg). The antinociceptive effects are antagonized by subcutaneous administration of naloxone (0.1–1 mg/kg) or nor-binaltorphimine (10–32 mg/kg). The sites of action of R-84760 in the formalin test have been suggested to be spinal and supraspinal, since opioid antagonists injected either intracerebroventricularly or intrathecally antagonize the antinociceptive effect of R-84760 (s.c.) (Fujibayashi and Iizuka, 1995; Fujibayashi et al., 1996). It was also shown that the descending noradrenergic neurons projecting to the spinal cord may play a role, at least partial, in the antinociceptive effect of R-84760 (Fujibayashi and Iizuka, 1995).

An excellent preparation has been developed to observe the effect of analgesics in vitro by using the spinal cord of neonatal rats (Otsuka and Konishi, 1974). A slow ventral root potential is recorded from the ventral root after the mono- and polysynaptic reflex discharge following stimulation of the ipsilateral dorsal root of the same segment. The slow ventral root potential, a depolarization lasting for a few tens of seconds, is considered to be a nociceptive response, since it is reduced by tachykinin $NK_1$ receptor antagonists and opioid compounds (Yanagisawa et al., 1982, 1985; Akagi et al., 1985). In the present experiments, the analgesic action of R-84760 was investigated on the slow ventral root potential. R-84760 inhibited the slow ventral root potential without affecting the monosynaptic

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reflex and the inhibitory effect was irreversible. In vivo nociceptive responses of mice were also inhibited by R-84760 but the effect was fully reversible. The site of R-84760 action in vitro was suggested to be presynaptic.

## 2. Materials and methods

### 2.1. Electrophysiological experiments

Neonatal Wistar–Imamichi rats (the Imamichi Institute of Animal Reproduction, Ibaraki, Japan) aged 1–7 days were used. Preparation of the isolated spinal cord and extracellular recording were performed according to methods reported by Otsuka and Konishi (1974). The rats were decapitated under ether anesthesia and the spinal cord was isolated and hemisected sagittally with the dorsal and ventral roots of the L3 and L4 segments attached. The preparation was placed in a recording chamber of 0.2 ml volume which was perfused continuously (2 ml/min) with an artificial cerebrospinal fluid (CSF) of the following composition (mM): NaCl, 138.6; KCl, 3.35; $MgCl_2$, 2.0; $CaCl_2$, 1.26; $NaH_2PO_4$, 0.58; $NaHCO_3$, 21.0 and glucose, 10.0. The artificial CSF was kept at $27 \pm 1$°C and saturated with 95% $O_2$ + 5% $CO_2$. Electrical stimulation (a single rectangular pulse of 0.3 ms and supramaximal intensity) was applied to the L3 or L4 dorsal root every 2 min and resultant mono- and polysynaptic reflex discharge and slow ventral root potential were recorded from the corresponding ventral root. In some experiments, the dorsal root potential was recorded from the dorsal root which was next to the one stimulated. The extracellular recording from the ventral or dorsal roots was performed by using a suction electrode (Otsuka and Konishi, 1974; Saito, 1979). Membrane potential changes in the ventral and dorsal roots were led to an analogue chart recorder through a preamplifier. The outputs from the preamplifier were led to a memory oscilloscope and a digital chart recorder. The magnitude of the monosynaptic reflex, slow ventral root potential and dorsal root potential was determined as the area between the basal d.c. level and the respective waveform and expressed as a percentage of the control value which was the mean of 4 responses immediately before the application of drugs. To obtain concentration–response curves, drugs such as R-84760 were applied cumulatively for 20 min each, and the mean value of the last 4 responses in each period was determined. Concentrations of drugs producing 50% inhibition ($IC_{50}$ values) were determined by linear regression analysis. Depolarizations induced by substance P, L-glutamate and γ-aminobutyric acid (GABA) were also recorded from the ventral root by applying the drugs to the perfusing solution for 3 or 10 s.

### 2.2. Antinociceptive tests

The tail pinch and tail flick tests in mice (ddY mice weighing 18–25 g; Japan SLC, Shizuoka, Japan) were performed using methods based on those of Takagi et al. (1966) and D'Amour and Smith (1941), respectively. In both tests, the cut-off time was set at 6 s and the absence of nociceptive behaviors within this time after the administration of R-84760 was defined as the antinociceptive response. The behaviors considered as nociceptive responses were biting a clip for pinch application, squeaking in the tail pinch test and flicking the tail in the tail flick test.

All experiments were carried out according to the guidelines provided by the Institutional Animal Care and Use Committee of Sankyo Co. (Tokyo, Japan).

### 2.3. Drugs

Drugs used were R-84760, (3*S*)-3-(1-pyrrolidinylmethyl)4-[(1*R*)-5,6-dichloro-1-indancarbonyl]-2,3,5,6tetrahydro-1,4-thiazine hydrochloride (R-86428, an enantiomer of R-84760) and nor-binaltorphimine dihydrochloride (synthesized in the Medicinal Chemistry Research Laboratories, Sankyo Co., Tokyo); U-50488 methanesulfonate, L-glutamate and GABA (Sigma Chemical Co., St. Louis, MO); [MeTyr$^1$, MeArg$^7$, D-Leu-NHEt$^8$]dynorphin A-(1–8) (E-2078), [D-Ala$^2$, MePhe$^4$, Gly-ol$^5$]enkephalin (DAMGO), [D-Pen$^{2,5}$]enkephalin (DPDPE) (Bachem Feinchemikalien, Bubendorf, Switzerland); substance P (Peptide Institute, Osaka, Japan); naloxone hydrochloride and tetrodotoxin (Sankyo Co., Tokyo).

## 3. Results

### 3.1. Effect on slow ventral root potential

The slow ventral root potential was depressed by R-84760 (Fig. 1) in 53 of 62 preparations, as observed previously with morphine or various tachykinin $NK_1$ receptor antagonists (Yanagisawa et al., 1985; Otsuka and Yanagisawa, 1988; Nussbaumer et al., 1989; Hosoki et al., 1994; Guo et al., 1995). The magnitude of the slow ventral root potential was reduced to $35 \pm 4\%$ (mean ± S.E.M., $n = 9$) of the control value after application of R-84760 (10 nM) for 60 min (Fig. 2). In the other 9 preparations, the slow ventral root potential was not affected by R-84760. The dorsal root potential was reduced in magnitude with R-84760 at 10 nM to $78 \pm 5\%$ ($n = 5$) of the control within 60 min after the start of application. On the other hand, the magnitude of the monosynaptic reflex was reduced by less than 10% of the control in 27 out of 32 preparations. In the rest, the monosynaptic reflex was attenuated to 80–90% of the control independently of the slow ventral root potential depression, i.e. attenuation began 30–40 min after the beginning of the slow ventral root potential depression. These results suggest that the decrease of the monosynaptic reflex was not caused by R-84760.

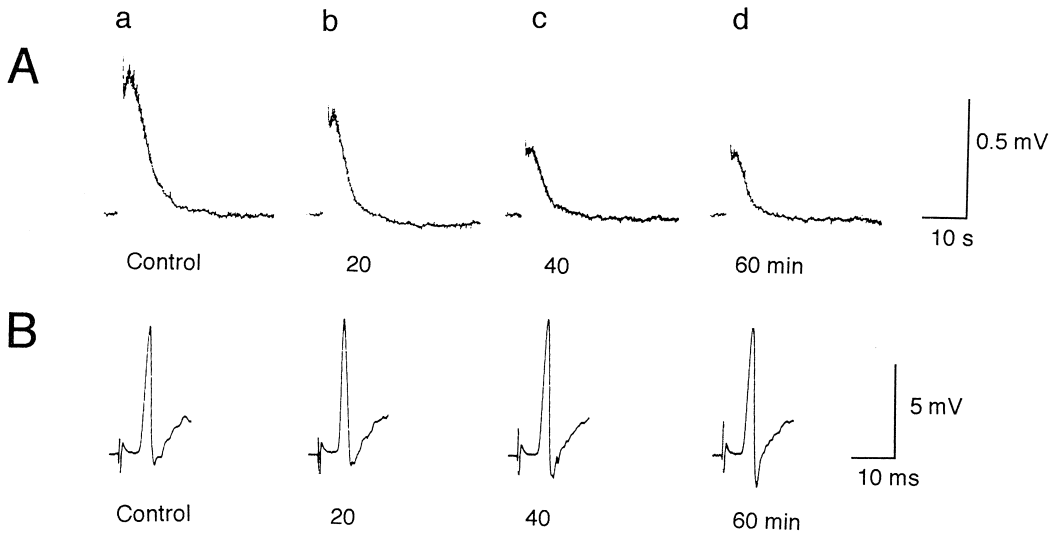


Fig. 1. Effect of R-84760 on the slow ventral root potential (A) and monosynaptic reflex (B) recorded from the L3 ventral root. (a) Control responses. (b)–(d) Responses recorded at the indicated time after the start of R-84760 (10 nM) application.

The effect of R-84760 on the slow ventral root potential was characterized by its time course, i.e. the depressant effect progressed slowly and reached a maximum about 60 min after the start of application, and recovered slightly within 2–5 h after removal of R-84760 (Fig. 2). Additionally, the time course and maximal level of depressant action of R-84760 were independent of the application time when the same concentration was employed, e.g. almost the same effect was obtained with 6- as with 60-min application of 10 nM R-84760 as shown in Fig. 2.

An inhibitory action on the slow ventral root potential was also observed with other $\kappa$- (U-50488 and E-2078), $\mu$- (DAMGO) and $\delta$- (DPDPE) opioid receptor agonists. Both development and recovery of the effect of these opioids were more rapid than those with R-84760. In particular, E-2078 and DAMGO, peptide agonists, induced their maximal effect as early as 15 min after the start of application and recovery began immediately after removal from the perfusing solution as shown in Fig. 2.

R-84760 was the most potent in reducing the slow

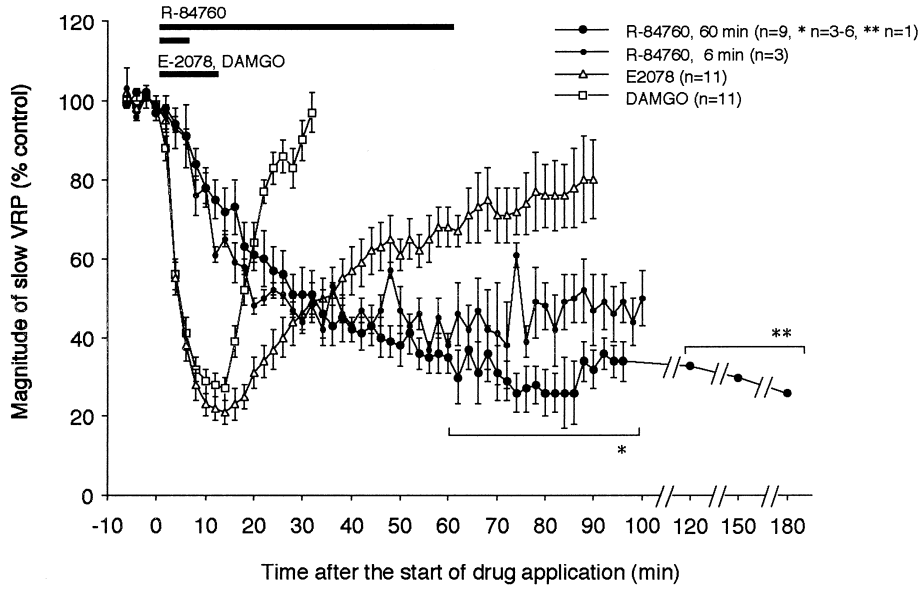


Fig. 2. Time courses of the effects of R-84760 (10 nM), E-2078 (100 nM) and DAMGO (10 nM) on the slow ventral root potential (VRP). R-84760 was applied for 6 and 60 min and E-2078 and DAMGO for 12 min as indicated by horizontal bars. The magnitude of the slow ventral root potential was expressed as a percent of the control value. Each symbol with a vertical bar represents the mean ± S.E.M. The number of preparations is shown in parentheses after each drug name.

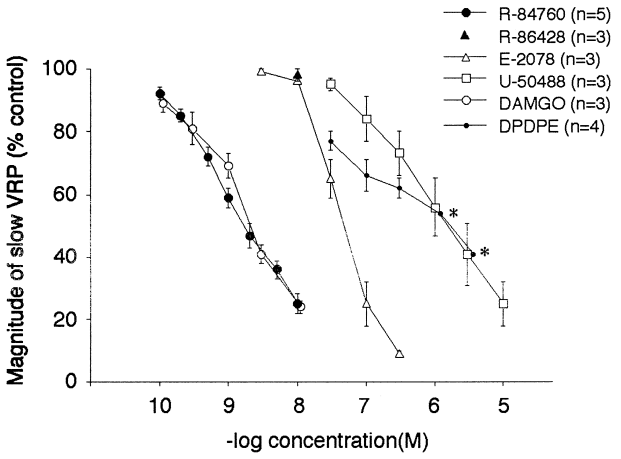


Fig. 3. Concentration–response curves of R-84760 and other opioid compounds for the inhibitory effect on slow ventral root potential (VRP). The magnitude of the slow ventral root potential was expressed as a percent of the control value. Each symbol with a vertical bar represents the mean ± S.E.M. The number of preparations is shown in parentheses except for the symbols with * where $n = 2$.

ventral root potential among the 5 opioids examined (Fig. 3) and the potency order was as follows: R-84760 ($IC_{50}$; 1.9 nM) ≥ DAMGO (2.0) > E-2078 (53) > DPDPE (1200) ≥ U-50488 (1500). R-86428, an enantiomer of R-84760, did not affect the slow ventral root potential at 10 nM (Fig. 3), suggesting that the effect of R-84760 is stereoselective.

### 3.2. Site of action of R-84760

In order to investigate whether R-84760 acts at pre- or postsynaptic sites, the effect of the drug on depolarizations induced by substance P and L-glutamate, both transmitters of the primary afferent C fiber, and GABA were investigated. R-84760 (10 nM) attenuated the depolarizations caused by substance P (in all of the 9 preparations examined) and L-glutamate (4 of 6) (Fig. 4), whereas those induced by GABA were hardly affected ($n = 3$). In order to eliminate a possible presynaptic component of R-84760 action, the effect of R-84760 on depolarizations was tested in the presence of tetrodotoxin. Under conditions such that mono- and polysynaptic reflex discharge and the slow ventral root potential were abolished in the presence of 0.3 μM tetrodotoxin, the depolarizations were not attenuated by R-84760 (Fig. 4; $n = 4$, 2 and 2 for substance P, L-glutamate and GABA, respectively). These results obtained in the presence and absence of tetrodotoxin suggest that R-84760 depresses the slow ventral root potential through a presynaptic action rather than a postsynaptic action in the synapse between the interneuron and motoneuron.

### 3.3. Effect of opioid receptor antagonists

The inhibition of the slow ventral root potential induced by DAMGO was abolished by naloxone, a relatively selective μ-opioid receptor antagonist, at 100 nM (Fig. 5). In contrast, the inhibition induced by R-84760 was reversed slightly by naloxone at 100 nM and substantially at 1 μM (Fig. 5). The inhibitory effects of R-84760 and DAMGO were also reduced or abolished by nor-binaltorphimine, a selective κ-opioid receptor antagonist (Fig. 5). R-84760

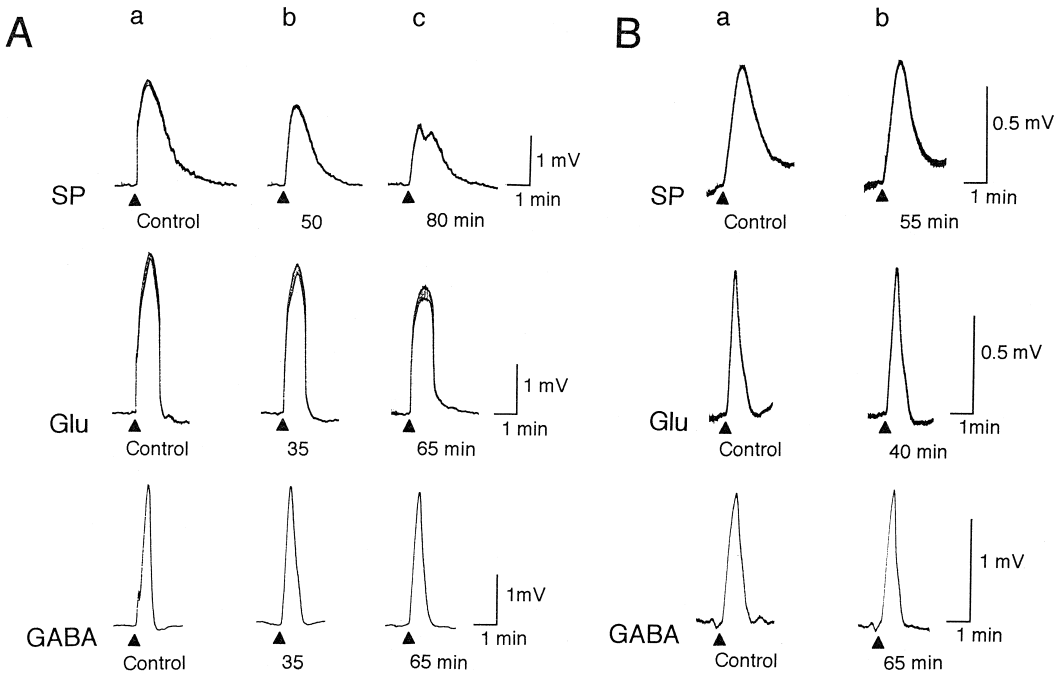


Fig. 4. Effect of R-84760 on depolarizations recorded from the ventral root with application of substance P (SP), L-glutamate (Glu) and GABA in the absence (A) or presence (B) of tetrodotoxin (0.3 μM). (a) Control responses. (b and c) Responses recorded at the indicated time after application of R-84760 (final concentration of 10 nM). Perfusing solution was exchanged with that containing substance P ($10^{-5}$ M), L-glutamate ($10^{-2}$ M) or GABA ($10^{-3}$ M) for 3 s except for GABA in B where it was exchanged for 10 s.

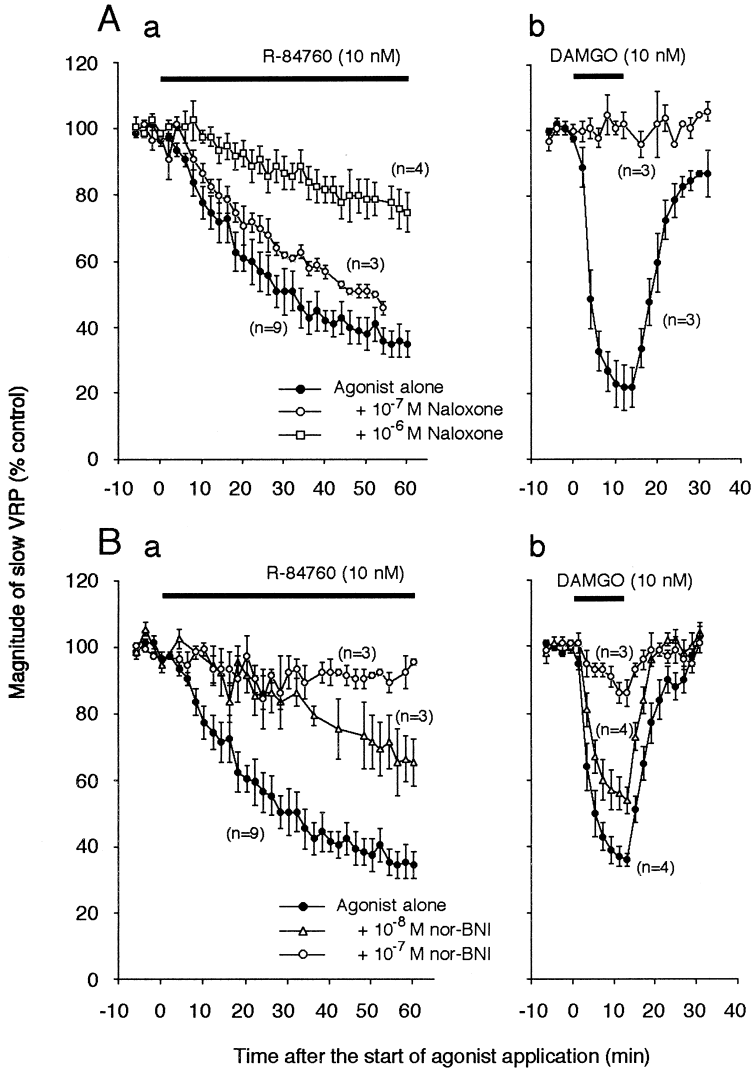


Fig. 5. Antagonism by naloxone (A) and nor-binaltorphimine (nor-BNI; B) of inhibitory action of R-84760 (a) and DAMGO (b) on the slow ventral root potential (VRP). The magnitude of the slow ventral root potential was expressed as a percent of the control value. Agonists were applied as indicated by solid bars and antagonists were applied throughout the observation periods. Each symbol with a vertical bar represents the mean ± S.E.M. The number of preparations is shown in parentheses. The values of filled circles in (A)a and (B)a are interchangeable and also with large filled circles up to 60 min in Fig. 2.

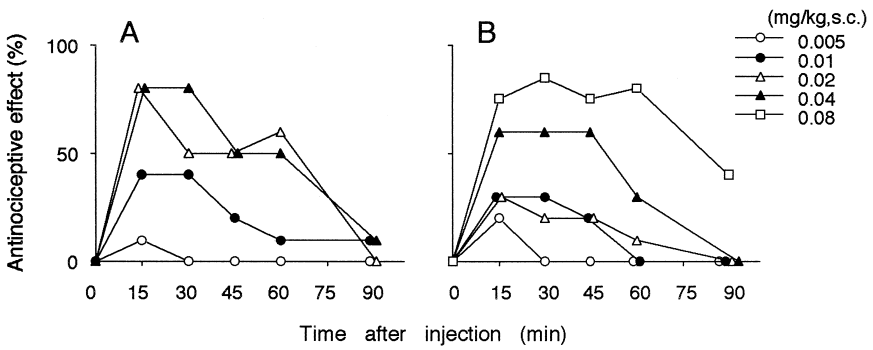


Fig. 6. Time courses of the antinociceptive effects of R-84760 in the tail pinch (A) and tail flick tests (B). The effects are expressed as percentages of mice which showed the antinociceptive response. 10 to 20 mice were used for each dose.

was more sensitive to nor-binaltorphimine than was DAMGO. The results suggest that R-84760 acts at the κ-opioid receptor.

### *3.4. Time course of the antinociceptive effects of R-84760 in mice*

The effect of R-84760 to reduce the slow ventral root potential lasted for an extended period without any sign of recovery. The time course of the antinociceptive effect of R-84760 in vivo was, therefore, examined in mice. Subcutaneous injections of R-84760 dose dependently induced the antinociceptive effects in the tail pinch and tail flick tests in mice (Fig. 6). The effects reached their maximum between 15 and 30 min after R-84760 administration, and disappeared during the next 60 min in most cases.

## 4. Discussion

R-84760 depressed the slow ventral root potential, known as nociceptive reflex discharge, whereas the monosynaptic reflex was hardly affected. Opioid receptor antagonists, naloxone and nor-binaltorphimine, antagonized the effect of R-84760. The subtype of opioid receptor which mediates the effect of R-84760 seems to be the κ- rather than the μ-type because a higher concentration of naloxone was required to antagonize the effect of R-84760 than of DAMGO. Higher sensitivity of R-84760 than of DAMGO to nor-binaltorphimine further showed that the effect of R-84760 is elicited through the κ-opioid receptor, although the selectivity of nor-binaltorphime for the κ-opioid receptor seems to be lower than that shown in our previous in vivo experiments in mice (Fujibayashi et al., 1994; Fujibayashi and Iizuka, 1995). A similarly poor selectivity of nor-binaltorphimine is shown after subcutaneous injection in mice (Endoh et al., 1992), or intrathecal injection in rats (Guirimand et al., 1994). A possible contribution of the δ-opioid receptor to the R-84760 effect can be excluded because R-84760 has only slight affinity for the δ-opioid receptor ($IC_{50}$, 1030 nM; Fujibayashi et al., 1994), and because the δ-opioid receptor is reported to be absent in the neonatal rat spinal cord (James et al., 1990). The inhibitory action of DPDPE on the slow ventral root potential may be due to a non-selective action through the μ- (James et al., 1990) or κ-opioid receptor.

The site of action of R-84760 for inhibition of the slow ventral root potential was elucidated by observing the effect of R-84760 on depolarizations induced by substance P, L-glutamate and GABA. The inability of R-84760 to attenuate the depolarizations induced by all three of these substances in the presence of tetrodotoxin indicates that R-84760 does not act postsynaptically on the motoneurons. The reduction by R-84760 in normal artificial CSF of the substance P- or L-glutamate-induced depolarization thus indicates that R-84760 inhibits transmission presynaptically from interneuron to motoneuron. The presynaptic action of R-84760 may be valid for other synapses, e.g. those between interneurons, and between the primary afferent C fiber and interneuron. In previous reports, postsynaptic (Besse et al., 1990; Kolaj et al., 1995; Randić et al., 1995) as well as presynaptic sites (Allerton et al., 1989; Besse et al., 1990; Randić et al., 1995) were postulated for the action of κ-opioids in the spinal dorsal horn neurons. The present results do not exclude a possible postsynaptic action of R-84760 on the interneurons. The lack of susceptibility of the GABA-induced depolarization to R-84760, irrespective of the presence or absence of tetrodotoxin, can be explained by a direct action of GABA on the motoneurons.

The inhibitory action of R-84760 on the slow ventral root potential progressed more slowly than that of DAMGO and did not recover after removal of R-84760 from the perfusing solution. The time course of R-84760 action was distinct from those of the antinociceptive effects of R-84760 shown after subcutaneous injections in mice. The time course of the inhibitory effect on the slow ventral root potential seems to be characteristic of neonatal rat spinal cord preparations. The action of E-2078, another κ-opioid receptor agonist, had a time course different from that of R-84760, suggesting that the slow action is a characteristic of R-84760 rather than a common property of κ-opioid receptor agonists. The irreversibility of the R-84760 effect could not be explained by a possible toxic effect because the monosynaptic reflex was intact. Mechanisms relating to the ligand–receptor interaction or intracellular messenger systems distinct from those of other opioid agonists may underlie the inhibitory action of R-84760.

In summary, R-84760, a selective κ-opioid receptor agonist, showed a specific inhibitory action on the nociceptive reflex in the isolated neonatal rat spinal cord, through the κ-opioid receptor. The slow progress and irreversibility of R-84760 action are thought to be characteristic of the drug in this preparation. The site of action is suggested to be presynaptic.

## Acknowledgements

The authors are grateful to Dr. Yoshio Iizuka for reading the manuscript and making suggestions.

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