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Effects of an intrathecally administered benzodiazepine receptor agonist, antagonist and inverse agonist on morphine-induced inhibition of a spinal nociceptive reflex

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1 The effects of an intrathecally administered benzodiazepine receptor (BZR) agonist (midazolam, up to 50 μg), antagonist (flumazenil, Ro 15–1788, 5 μg) and inverse agonist (Ro 19-4603, 15 μg) on nociception and on morphine-induced antinociception were studied in rats.

2 By themselves, none of these compounds significantly altered pain threshold.

3 The BZR agonist midazolam enhanced the morphine-induced antinociceptive effect whereas the antagonist flumazenil did not alter it. In contrast, the BZR inverse agonist Ro 19-4603 decreased the morphine-induced antinociceptive effect.

4 Naloxone (1 mg kg^{-1} i.p.) completely reversed all these effects.

5 These results demonstrate that BZR agonists and inverse agonists are able to affect, by allosteric up- or down-modulation of γ-aminobutyric acid$_A$ (GABA$_A$)-receptors, the transmission of nociceptive information at the spinal cord level, when this transmission is depressed by μ-opioid receptor activation.

Introduction

Several benzodiazepine derivatives have been found to reduce responses to noxious stimuli (Haefely *et al.*, 1981). This reduction can be most easily explained by their sedative/tranquillizing properties, resulting from an action at the supra-spinal level.

However, it is well known that part of the anti-nociceptive effect of different compounds originates within the spinal cord. For example, intrathecal administration of morphine inhibits nociceptive motor responses (Yaksh & Rudy, 1976b; 1977) and activity in ascending axons evoked by stimulation of nociceptive afferent nerve fibres (Doi & Jurna, 1982).

Benzodiazepine receptor (BZR) ligands are also able to act at the spinal cord level. BZR agonists have been shown to enhance dorsal root potentials, indicating an increased depolarization in those primary afferents that mediate presynaptic inhibition of motoneurones (Polc *et al.*, 1974). Diazepam reduces polysynaptic, and less consistently, monosynaptic reflex activity. Reflex inhibition was directly related to the increase in primary afferent depolarization induced by diazepam (Stratten & Barnes, 1971).

In addition to these effects, benzodiazepine derivatives may also reduce impulse transmission from primary nociceptive afferents to spinal neurones that send their axons to the brain. Indeed, the intrathecal (i.t.) injection of diazepam was shown to depress the activity in ascending axons of the spinal cord, evoked by electrical stimulation of afferent C fibres (Jurna, 1984). Furthermore, the i.t. administration of midazolam was found to depress nociceptive sympathetic reflexes (Whitwam *et al.*, 1983; Niv *et al.*, 1983) and to produce a selective sensory blockade, abolishing pain of somatic origin (Goodchild & Noble, 1987). These observations suggest a spinal site of action for the modification of nociceptive information by benzodiazepines.

The present study was carried out to investigate whether benzodiazepine receptor (BZR)-ligands interact with opiate mechanisms mediating analgesia at the spinal cord level.

The tail-flick paradigm was chosen for evaluating anti-nociceptive effects since the tail flick response is considered to be a predominantly spinal event.

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As a representative benzodiazepine receptor agonist, we chose midazolam, since this compound as a salt is water-soluble, has a rapid onset and a short duration of action and because the activity of its metabolites is negligible (Pieri *et al.*, 1981; Pieri, 1983). We also tested the effects of i.t. administration of a benzodiazepine receptor antagonist (flumazenil, Ro 15-1788; Hunkeler *et al.*, 1981) and of a potent benzodiazepine receptor inverse agonist, Ro 19-4603 (Kyburz, 1986), on morphine-induced antinociception.

Methods

Animals

Experiments were carried out on 28 male SPF-Fü rats (350–400 g) kept on a 12 h-light/12 h-dark cycle and housed in individual cages with food and water *ad libitum*.

Surgical and histological procedures

Each animal was anaesthetized with sodium pentobarbitone (50 mg kg^{-1} i.p.) and placed into a stereotaxic apparatus with the head flexed forward. A polyethylene tube (PE 10) was inserted through a slit in the cisternal membrane 8.5 cm down the spinal subarachnoid space to the rostral aspect of the lumbar enlargement according to the method of Yaksh & Rudy (1976a). The catheter was fixed to the skull with stainless steel screws and cranioplastic cement. Animals were then allowed to recover for at least 5 days. The animals which exhibited motor deficits as a result of surgery were discarded from the study.

After completion of the experiments, an i.t. injection of 5 μl methylene blue followed by 7 μl balanced ion solution was made. All animals were then killed with an overdose of sodium pentobarbitone and intracardially perfused with 0.9% w/v NaCl solution followed by 10% formalin. The spinal cord was then dissected and the localization of the catheter's tip verified.

Experimental protocol

The animal's responsiveness to noxious thermal stimuli was determined using the tail-flick test (D'Amour & Smith, 1941). A high intensity beam of light from a projector lamp was focused on the dorsal surface of the rat's tail. The number of seconds (precision: 0.01 s) that elapsed until a reflex movement of the tail from the path of the beam occurred was automatically determined. Tail temperature was not routinely monitored, instead, the lamp's intensity was set so that the baseline tail-flick (TF) latency was 3.5–4.5 s. A cut-off latency of 10 s was used to avoid skin damage.

After baseline TF latency had been determined, the rats were injected i.t. with drugs in a volume of 5 μl followed by 7 μl of balanced ion solution to flush the catheter. Injections were delivered over a period of about 20 s. The balanced ion solution consisted of (in g l^{-1} distilled water) NaCl 7.46, KCl 0.19, $MgCl_2$ 0.19 and $CaCl_2$ 0.14 (Yaksh & Rudy, 1976a). TF latency was measured 15, 30, 45 and 60 min and, in some cases, also 65 and 75 min after injection. A delay of at least 4 days separated consecutive experiments in any given animal.

The following drugs were administered intrathecally: midazolam hydrochloride (16.5–50 μg), Ro 19–4603 free base ((tert-butyl 5,6-dihydro-5-methyl-6-oxo-4H-imidazo [1,5-a] thieno [2,3-f][1,4] diazepine-3-carboxylate), 15 μg), flumazenil free base (Ro 15-1788, ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo [1,5a][1,4] benzodiazepine-3-carboxylate, 5 μg), which were synthesized in our research department, and morphine hydrochloride (3–15 μg), which was purchased from Siegfried AG, Switzerland.

To compare the different treatments performed in the same rats, statistical analysis was carried out by use of a paired Student's *t* test (Glantz, 1981).

Results

On the basis of the histological verification, two out of 28 animals were discarded: in the first one the catheter was located in the central canal of the spinal cord and in the second one the catheter's tip was clogged by fibrosis. In all other animals, the catheter tip was correctly placed and open and drug injections never produced any gross motor impairment or any significant histological defect.

Effect of intrathecal injection of midazolam on the anti-nociceptive action of morphine

Figure 1 represents the variations of tail-flick (TF) latencies as a function of time after i.t. administration of different compounds. As previously shown (Yaksh & Rudy, 1977), morphine produced a dose-dependent increase in the mean TF latency; after 15 μg, TF latency increased from baseline values of 4 to 4.5 s to the cut-off value of 10 s within about 30 min, while after 3 μg it reached an intermediate level of about 7.5–8 s. Midazolam alone (16.5 μg and

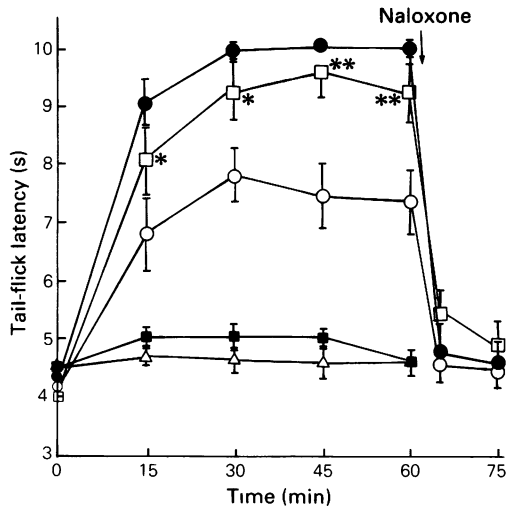

Figure 1 Antinociceptive effect of intrathecal injection of morphine (●, 15 μg; ○, 3 μg), midazolam (■, 16.5 μg), and morphine (3 μg) + midazolam (16.5 μg) simultaneously administered (□). One hour after intrathecal infusion, naloxone (1 mg kg^{-1}, i.p.) was injected. Controls (△). Points represent mean of 12 observations and vertical lines indicate s.e. mean. The morphine 3 μg group compared to the morphine 3 μg + midazolam 16.5 μg group: * $P \leqslant 0.05$; ** $P \leqslant 0.01$.

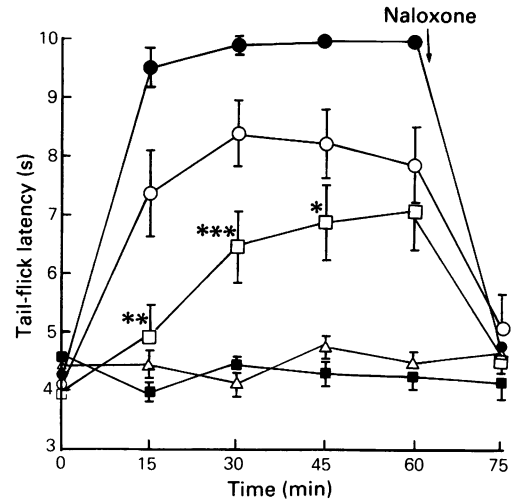

Figure 2 Antinociceptive effect of intrathecal injection of morphine (●, 15 μg; ○, 3 μg), Ro 19-4603 (■, 15 μg), and morphine (3 μg) + Ro 19-4603 (15 μg) simultaneously administered (□). One hour after intrathecal infusion, naloxone (1 mg kg^{-1} i.p.) was injected. Controls (△). Points represent mean of 14 observations and vertical lines indicate s.e. mean. The morphine 3 μg group compared to the morphine 3 μg + Ro 19-4603 15 μg group: * $P \leqslant 0.05$; ** $P \leqslant 0.01$; *** $P \leqslant 0.001$.

50 μg, the latter not shown on the figure) did not significantly increase the TF latency. When injected simultaneously with 3 μg of morphine, midazolam induced a statistically significant increase in morphine-antinociception. Note that all of these effects were reversed by naloxone 1 mg kg^{-1}, i.p.

Effect of intrathecal injection of Ro 19-4603 on the antinociceptive action of morphine

Figure 2 illustrates the effect of Ro 19-4603 on morphine-induced antinociception. Once again, morphine produced a dose-dependent increase in the mean TF latency. Ro 19-4603 alone (15 μg) produced a slight decrease of TF latency 15 min after injection which was of borderline statistical significance, but in general did not significantly modify the TF reflex. When injected together with 3 μg of morphine, Ro 19-4603 significantly decreased the antinociceptive effect of morphine for at least 45 min. These effects were subsequently reversed by naloxone 1 mg kg^{-1} i.p.

Effects of intrathecal injection of flumazenil on the antinociceptive action of morphine

The i.t. injection of flumazenil (5 μg) neither reduced nor enhanced the antinociceptive action of morphine (3 μg) (data not shown). Neither flumazenil nor vehicle alone modified the tail-flick latency.

Discussion

Our results demonstrate that intrathecal (i.t.) injections of some BZR-ligands are able to modify morphine-induced antinociception in the rat as assessed by the TF response. The first experiment showed that midazolam (a benzodiazepine agonist) was able to potentiate the antinociceptive effect of morphine in doses that alone had no significant effect. In contrast, the second experiment showed that Ro 19-4603 (a benzodiazepine receptor inverse agonist) was able to decrease the antinociceptive effect of morphine. The fact that an equivalent volume of vehicle injected into the intrathecal space at the same spinal level failed to affect the TF reflex, and that two different compounds act in opposite directions, indicate that the observed results were not due to any non-specific effects such as mechanical pressure.

As is well known, BZR ligands exert their effects by interacting with the γ-aminobutyric acid (GABA) system (Haefely *et al.*, 1975; Costa *et al.*, 1975; Haefely & Polc, 1986). Thus, the present results are in good agreement with previous studies that have

shown that morphine analgesia can be enhanced by increasing the concentration of GABA in the CNS (Contreras *et al.*, 1979; Buckett, 1980) or by administration of the GABA agonist muscimol (Biggio *et al.*, 1977). However, these studies did not involve local application and the results are contradicted by other studies (Christensen *et al.*, 1978). It appears therefore that the route of administration and the number of GABAergic synapses affected are critical in determining the direction of the GABA-morphine interaction (see Sawynok, 1984). It has also been shown that GABA itself possesses analgesic properties (Buckett, 1980). However, neither midazolam (up to the very high concentration of $50\,\mu g\,5\,\mu l^{-1}$, i.e. $27.6\,mmol\,l^{-1}$, a concentration which has been shown to reduce the conduction of action potentials in the rat isolated sciatic nerve, Pieri *et al.*, 1981) nor Ro 19-4603 administered alone were able to alter significantly the TF reaction time.

In contrast to our present observation, Niv *et al.* (1983) found that i.t. midazolam had analgesic properties which were not reversed by naloxone, and they suggested that the antinociceptive effect of locally applied midazolam could be the result of an effect on a non-opioid GABA-mediated system. The discrepancy between our results and those of Niv might well be due to experimental differences. Indeed, there are many procedural differences between the two studies with regard to the animal species used (anaesthetized dog vs freely behaving rat) and the method of assessment of the effectiveness of drugs on nociceptive stimulation (evoked sympathetic reflex vs tail-flick reflex). This discrepancy might be the result of a species difference (antinociceptive mechanisms might not necessarily be the same in dog and rat). It might also be explained by the fact that Niv used the anaesthetized dog and the anaesthetic might have increased or decreased some endogenous mechanism involved in the control of nociceptive information.

The lack of effect of midazolam and Ro 19-4603 injected alone can be explained in different ways. Firstly, it is possible that, under normal circumstances, the GABA system does not tonically modulate the nociceptive pathways. Alternatively, the well-known descending inhibitory system controlling nociceptive transmission at the spinal level (Basbaum & Fields, 1984) could be tonically inhibited at the supraspinal level, counteracting an antinociceptive action through GABA synapses at the spinal cord level. Thirdly, in a recent paper, Stein and colleagues (1987) have shown that i.t. injection of pentobarbitone, a barbiturate also acting at the level of the $GABA_A$ receptor complex, did not change TF reflex in animals with intact neuraxis, but did produce a significant elevation in TF latencies in spinal cord transected animals. From this observation they proposed the existence in the intact CNS of a tonic descending excitatory system that would neutralize a GABA-mediated antinociceptive action visible only in the spinal animal. The existence of such a system could also explain why BZR ligands (also acting at the $GABA_A$ receptor complex) have no significant effect when injected i.t. in intact animals. More detailed pharmacological analysis remains to be done to specify further the relative involvement of the different components in the modulation of nociception at the spinal level.

When administered intrathecally in combination with a low dose of morphine, midazolam (a BZR agonist) enhanced morphine-induced antinociception. The fact that Ro 19-4603, a BZR inverse agonist, acted in the opposite direction on this morphine effect is, in our opinion, clear-cut evidence for the involvement of the benzodiazepine receptor in the effects of these BZR ligands. These findings are in good agreement with clinical data showing that the combined use of midazolam and an opiate results in an enhancement of opiate analgesia (Forster *et al.*, 1980; Gemperle & Kapp, 1983).

Anatomical studies have revealed an overlap in the distribution of opiate and benzodiazepine receptors in the spinal cord. Indeed both opiate and benzodiazepine receptors have been shown to be particularly concentrated in the dorsal horn of the spinal cord (especially in the substantia gelatinosa) (Atweh & Kuhar, 1977; Richards *et al.*, 1986). It has also been shown that, following i.t. injection of $5\,\mu l$ of radiolabelled morphine, the predominant levels of radioactivity remained in the vicinity of the catheter tip (Yaksh & Rudy, 1977). We can speculate that the diffusion of intrathecally administered BZR ligands would be of the same order of magnitude and thus, all these observations argue in favour of a very localized, specific effect of BZR ligands at the spinal level.

In conclusion, these results indicate that BZR agonists and inverse agonists are able, by allosteric modulation of $GABA_A$ receptors, to affect the transmission of nociceptive information at the spinal cord level when this transmission is depressed by opioid receptor activation.

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