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The spinal potentiating effect and the supraspinal inhibitory effect of midazolam on opioid-induced analgesia in rats

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Abstract

The authors investigated the effects of spinal and supraspinal administration of the benzodiazepine receptor agonist midazolam alone and with opioids on tests of nociception (tail-flick and hot-plate tests) and motor function (catalepsy) in rats. At the spinal level, the dose-response curves for peak effect and area under the curve for morphine were shifted to the left (indicating potentiation) by a submaximal dose of intrathecal (i.t.) midazolam (20 μg) in both nociceptive tests. Additionally, 2.5 μg of i.t. midazolam, a dose having no effect when given alone, increased antinociception in both tests when given with i.t. morphine. Isobolographic analysis confirmed that i.t. injection of midazolam potentiated antinociception induced by i.t. morphine. At the supraspinal level, intracerebroventricular (i.c.v.) injection of 4 μg of midazolam inhibited morphine antinociception, i.e., the dose-response curve for morphine in the hot-plate test shifted to the right. Midazolam did not affect morphine antinociception in the tail-flick test. Catalepsy occurred only when the highest doses of i.t. or i.c.v. morphine or midazolam were injected alone. The differing effect of midazolam on morphine-induced antinociception suggests that different mechanisms are involved in the spinal cord and brain.

Keywords: Antinociception; (Intracerebroventricular); (Intrathecal); Benzodiazepine; Nociceptive test; Opioid

1. Introduction

The use of benzodiazepine agonists with opioids has gained wide acceptance among anesthesiologists. However, contrary to the common clinical impression that benzodiazepines potentiate the analgesia produced by opioids, studies indicate that anxiolytics such as midazolam and diazepam diminish the effectiveness of opioids (Hall et al., 1974; Singh et al., 1981; McDonald et al., 1986; Luger et al., 1992).

In animals, intrathecal (i.t.) administration of midazolam and diazepam alone produces an antinociceptive effect that is probably mediated by the actions of those drugs on γ-aminobutyric acid (GABA)ergic neurotransmission (Niv et al., 1983,1988; Jurna, 1984; Goodchild and Serrao, 1987). Benzodiazepine agonists act through a benzodiazepine-specific receptor in a benzodiazepine receptor-GABA ionophore complex (Haefely and Polc, 1986; Martin, 1987; Möhler and Richards, 1988; Haefely, 1989) that may be involved in the processing of nociceptive impulses (Sawynok, 1987). In contrast, some authors found that i.t. midazolam only transiently increased or did not affect antinociception (tail-flick latencies) in rats (Moreau and Pieri, 1988; Yanez et al., 1990). Also, Niv et al. (1988) report that midazolam (administered by itself) has a hyperalgesic effect when given systemically, an effect Niv and colleagues believe results from actions of midazolam in the brain.

Similar contradictory reports exist regarding benzodiazepines given with opioids. Systemic administration of benzodiazepine agonists in animals has both decreased (Palaoglu and Ayhan, 1986a,b; Daghero et al., 1987; Rosland and Hole, 1990a) and increased (Morichi and Pepeu, 1979) antinociception produced by systemi-

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cally administered morphine. Diazepam given systemically antagonized systemic morphine analgesia in the formalin test but had no effect in the tail-flick test (Abbott and Franklin, 1986). Intracerebroventricular (i.c.v.) administration of benzodiazepine agonists may reduce antinociceptive effects of opioids given systemically (Mantegazza et al., 1982). However, benzodiazepine agonists administered i.t. potentiate opioid antinociception (Moreau and Pieri, 1988; Yanez et al., 1990). Some studies on i.t. injection of these drugs show a dual effect of midazolam that depends on the relative concentrations of the drugs (Tejwani et al., 1990; Rattan et al., 1991). On the other hand, when given alone, the antinociceptive action of midazolam seems to be a function of the spinal cord, and hyperalgesia can be observed when the drug reaches supraspinal levels, i.e., production of an analgesic vs. a hyperalgesic effect seems to depend on the route of administration (Niv et al., 1988).

Our study therefore investigated whether any positive or negative influence of midazolam on morphine-induced antinociception would depend on the route of administration or the concentration of the drugs. Sites of opioid action for the modification of nociceptive input seem to exist in the spine as well as the brain, and these two sites of opioid actions may be influenced in opposite ways by benzodiazepines, through GABAergic mechanisms. We thus evaluated the nature of the combined administration of benzodiazepine agonists and opioids at both spinal and supraspinal levels.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (250–320 g) were used for these experiments. After catheter implantation, all animals were housed in individual cages on a 12-h light/dark cycle at a constant temperature of 23 ± 1°C with free access to food and water. Animals were brought to the testing room on the morning of the day of testing; testing took place during the light cycle. The study protocol was approved by the Fred Hutchinson Cancer Research Center Institutional Review Board.

2.2. Implantation of catheters

Intrathecal and intracerebroventricular catheters

The rat was placed in a stereotaxic frame with the head flexed forward. Separate groups of rats had catheter implantation as follows. For implantation of an i.t. catheter, anesthesia was induced with halothane, and an 8.5-cm-long piece of PE-10 polyethylene tubing was inserted through a slit in the cisternal membrane and advanced to the rostral aspect of the spinal cord at the thoracolumbar level. The external part of the catheter was tunneled subcutaneously to exit at the top of the skull (Yaksh and Rudy, 1976).

For implantation of an i.c.v. guide (a thin-walled, 24-ga tube), the rat was anesthetized with Equithesin solution (0.33 ml/100 g body weight) intraperitoneally, and a hole was trephined through the skull at coordinates overlaying the left lateral ventricle – 1 mm from the bregma and 1 mm from the midline (as described by Paxinos and Watson, 1986). The guide was inserted into the lateral ventricle (3 mm from the skull) and was fixed by attachments to stainless steel screws placed in the skull bones. Animals were used 4–7 days later, for only one experiment. Rats having motor abnormalities were not studied.

Verification of catheter placement

After testing, and depending on the type of catheter inserted, each animal had either an i.t. (10 μl) or i.c.v. (5 μl) injection of brilliant green ink. Each injection was flushed with 10 μl of i.t. saline or 5 μl of i.c.v. saline, respectively. At the end of the experiment, the location of the distal end of the i.t. or i.c.v. catheter was verified by independent postmortem examination of the rats by two investigators. Each rat was examined under the dissecting microscope for the existence of dye in the spinal cord or cerebral ventricle. All catheters were found to be placed correctly.

2.3. Drug administration

For site-selective administration, morphine sulfate (doses calculated as base; Mallinckrodt, St. Louis, MO, USA) and midazolam (Hoffmann-La Roche, Nutley, NJ, USA) were administered alone or together by either i.t. or i.c.v. injection. Drugs for i.t. administration were dissolved in 0.9% sodium chloride, so that 10 μl contained the desired dose of the agents. After each injection, the catheter was flushed with an i.t. injection of 10 μl of 0.9% sodium chloride. Drugs for i.c.v. administration were dissolved in 0.9% sodium chloride, so that 5 μl contained the desired quantity of the agents for the total i.c.v. dose; 5 μl of 0.9% sodium chloride was used to flush the i.c.v. guide. Drugs were delivered by microinjector syringe over a period of 20 s.

For subseries 1, we administered morphine, midazolam and a combination of a 'submaximal dose' of midazolam (20 μg i.t. or 4 μg i.c.v.) with morphine. We defined the submaximal dose as being the highest dose administered without signs of catalepsy or motor dysfunction in two tests of nociception, the hot-plate and tail-flick tests.

In subseries 2, we compared the effects of saline, a 'minimal effective dose' of midazolam (2.5 μg i.t. or 4 μg i.c.v.), morphine (1 μg i.t. or 4 μg i.c.v.) and a

combination of midazolam and morphine (same doses). A minimal effective dose of midazolam was defined as being the dose producing only $\leq 15\%$ of the maximum possible effect (MPE) in the hot-plate and tail-flick tests.

For subseries 3 (isobolographic analysis), the relative potency of i.t. midazolam and morphine was calculated to be 1:13, using the '2 and 2' dose assay of Tallarida and Murray (1987) and nociceptive responses for these drugs in the tail-flick test in subseries 1. This method was used because only in the tail-flick test was a maximum response of more than 50% able to be measured. To perform isobolographic analysis, we injected midazolam and morphine as fixed ratios of the ED_{50} dose for each drug. That is, for each group, we used i.t. injection of four equieffective dose combinations not producing catalepsy or changes in motor function:

ED_{50} morphine + ED_{50} midazolam

$2/3\,ED_{50}$ morphine + $2/3\,ED_{50}$ midazolam

$1/3\,ED_{50}$ morphine + $1/3\,ED_{50}$ midazolam

$1/6\,ED_{50}$ morphine + $1/6\,ED_{50}$ midazolam

I.c.v. midazolam was found to have no effect on the hot-plate and tail-flick tests. Because calculations for the ED_{50} doses revealed no pharmacologically relevant values, we did not subject i.c.v. data to isobolographic analysis.

2.4. Behavioral tests

Time course

To permit adaptation to surroundings (preadaptation), we brought animals to the testing room in the morning and placed each rat on the unheated hot plate for 1–2 min. Two hours later, we obtained baseline measurements for nociceptive and motor function tests, followed immediately by drug administration. All tests were repeated 5, 15, 30, 60 and 90 min after injection of morphine and/or midazolam, alone or in combination, or the control substance (given in order to reveal possible learning or tolerance responses). Behavioral tests (e.g., the test for catalepsy) were performed independently by one of the investigators, who did not know which drug had been administered. Baseline measurements were obtained in the same order: observation of behavior, test for catalepsy, hot-plate test and tail-flick test); baseline tail-flick tests and baseline hot-plate tests were performed twice within 30 min.

Nociceptive tests

In the hot-plate test, animals were placed on a metal plate that was enclosed by Plexiglas walls and maintained at 52.5 ± 0.5°C. The behavioral end point was represented by the number of seconds until the animal jumped off the plate or licked a hind paw. To prevent tissue damage, the test was terminated at 60 s if the animal did not respond. Animals underwent only one trial per test and time interval.

In the tail-flick test, the tail of each animal was placed on a rectangular metal plate under an illuminated lamp (100 W), with the light beam focused 1–3 cm from the tip of tail. Two trials occurred per test and time interval, i.e., the light beam was focused 1.5 cm from the tip of the tail in one trial and at 3 cm in the second trial; the time interval was 10 s. The end point was represented by the number of seconds until the rat flicked its tail out of the beam. To avoid tissue damage, the test was terminated at 6 s if the animal did not respond.

Motor function tests

All rats were observed for motor function. 'Motor dysfunction' consisted of complete loss of hind-limb

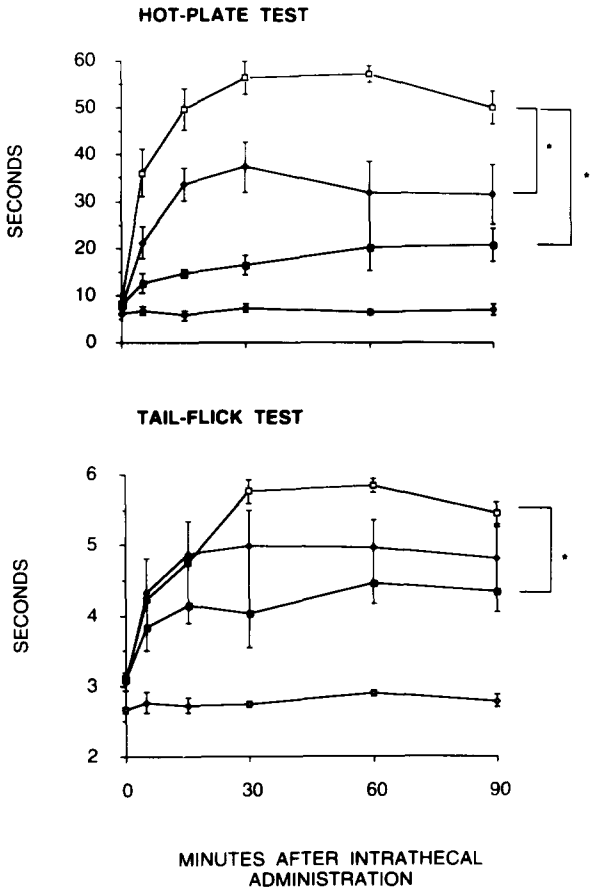

Fig. 1. Response times in two nociceptive tests plotted against time for randomly chosen doses of morphine (5 μg, $n = 6$, ◆), midazolam (20 μg, $n = 5$, ■), a combination of the two (same doses, $n = 7$, □) and saline ($n = 4$, ◇) injected intrathecally in rats. Co-administration of midazolam and morphine increased response latencies, demonstrating a potentiating effect of midazolam on morphine-induced antinociception. The cutoff time was 60 s for the hot-plate test and 6 s for the tail-flick test. As determined by analysis of variance with repeated measures, $^{*}P \leq 0.05$.

function. To evaluate catalepsy, we placed the arms of the animal on a 9-cm-high bar and measured the time to one of two responses: (1) climbing with both hind paws to the top of the bar, or (2) removal of both arms from the bar. Animals showing neither response within 30 s were given the maximal positive score for catalepsy (index of 1).

2.5. Data analyses

Examining the raw data for each animal separately, we calculated baseline, peak effect and time of peak effect for all animals per dose. To show drug effect as a value between the baseline value and the value at the cutoff time for the test, we expressed hot-plate, tail-flick and catalepsy response latencies as percentages of the maximum possible effect (%MPE) and area under the curve (AUC) for the effect vs. time relationship.

$$\%\text{MPE} = \frac{(\text{Post-drug time} - \text{pre-drug time})}{(\text{Cutoff time} - \text{pre-drug time})} \times 100$$

To compare the transformed data between treatment groups for subseries 1 and 2, transformed data were analyzed using the Kruskal-Wallis test. For evaluation of raw data pertaining to the randomly chosen interaction of 20 μg of i.t. midazolam and 5 μg of i.t. morphine as well as 4 μg of i.c.v. midazolam and 2 μg of i.c.v. morphine, we used analysis of variance (ANOVA) for overall treatment effects and applied Bonferroni corrections to P values. Statistical significance was accepted at $P \leq 0.05$.

Construction of the isobologram (subseries 3) for the drug-drug interaction followed the procedure of Tallarida et al. (1989). Data for individual and combination dose-response curves were used to generate the isobologram. Between the experimentally determined ED_{50} for morphine and midazolam, a theoretical additive line was drawn. Any point falling on this line indicates simple addition of effects of each drug. If the doses of the mixture turn out to be lower than those for additivity, potentiation or synergism is said to occur (Tallarida et al., 1989). Any point moved statistically to the right indicates antagonism between morphine and midazolam.

To perform isobolographic analysis, we evaluated dose-response curves for the groups given equieffective dose combinations not producing catalepsy, using regression lines based on the probit vs. log dose transformation for determining ED_{50} and 95% confidence intervals (Tallarida and Murray, 1987). For values of the probits corresponding to ≤ 0% and 100%, we used the method of Litchfield and Wilcoxon for confidence limits of ED_{50}s (Tallarida and Murray, 1987). Statistical significance was evaluated by comparison of the regression lines for additivity and the mixture to determine log (potency ratio) and the 95% confidence intervals of log ($\text{dose}_{\text{additivity}}/\text{dose}_{\text{mixture}}$). If the confidence limits did not contain zero, the potency ratio differed from 1, meaning that the mixture was significant (superadditive or subadditive) (Tallarida et al., 1989).

Table 1
Antinociceptive effects after intrathecal and intracerebroventricular administration of (1) a minimal effective dose of midazolam[a], (2) morphine and (3) the combination of morphine and a minimal effective dose of midazolam[a] in rats

Test	Saline	Midazolam [a] (2.5 μg)	Morphine (1 μg)	Midazolam [a] + morphine (2.5 μg + 1 μg)	P value
Intrathecal administration (n = 6–8 per dose)					
Hot-plate test					
%MPE	3.2 (±1.4) [b]	18.3 (±4.7)	31.1 (±6.3) [c]	96.53 (±3.5) [b,c]	0.0005
AUC	117.4 (±66.5) [b]	952.9 (±262.8)	1750.9 (±355.4) [c]	7057.9 (±587.6) [b,c]	0.0006
Tail-flick test					
%MPE	8.9 (±2.4) [b]	14.8 (±6.5) [b]	46.4 (±9.6) [c]	99.4 (±0.6) [b,c]	0.0008
AUC	450.3 (±145.6) [b]	869.1 (±403.9) [b]	3002.9 (±945.3) [c]	8186.0 (±270.3) [b,c]	0.0013
Test	Saline	Midazolam [a] (4 μg)	Morphine (4 μg)	Midazolam [a] + morphine (4 μg + 4 μg)	P value
Intracerebroventricular administration (n = 4–8 per dose)					
Hot-plate test					
%MPE	4.4 (±1.0) [b]	3.7 (±1.0) [b]	79.4 (±9.9)[c]	30.5 (±9.7) [b,c]	0.0005
AUC	197.3 (±57.5) [b]	167.3 (±61.0) [b]	4122.2 (±721.0) [c]	1608.6 (±602.1) [b,c]	0.0012
Tail-flick test					
%MPE	8.1 (±1.8) [b]	24.0 (±4.7) [b]	93.3 (±6.7) [c]	72.0 (±11.8) [c]	0.001
AUC	226.5 (±120.8) [b]	1121.8 (±416.0) [b]	7247.5 (±714.9) [c]	4844.1 (±952.7) [c]	0.0015

Antinociceptive effects (mean ± S.E.M.) are expressed as peak percentages of the maximum possible effect (%MPE) and area under the curve (AUC) for the effect-time relationship in the hot-plate and tail-flick tests. [a]The minimal effective dose of midazolam, i.e., the dose producing ≤ 15% of the maximum possible effect. Significances: $P \leq 0.05$ when compared with [b]morphine or [c]saline; Kruskal-Wallis test.

3. Results

3.1. Intrathecal administration

Nociceptive response

Fig. 1 shows the response times in nociceptive tests over 90 min for randomly chosen i.t. doses of morphine (5 μg), midazolam (20 μg) and a combination of the two (same doses). I.t. administration of 5 μg of morphine increased nociceptive response latency, the peak occurring 40.8 ± 8.4 min after drug injection in the hot-plate test and 40.0 ± 6.3 min after drug injection in the tail-flick test. Co-administration of 20 μg of i.t. midazolam significantly increased response latency to

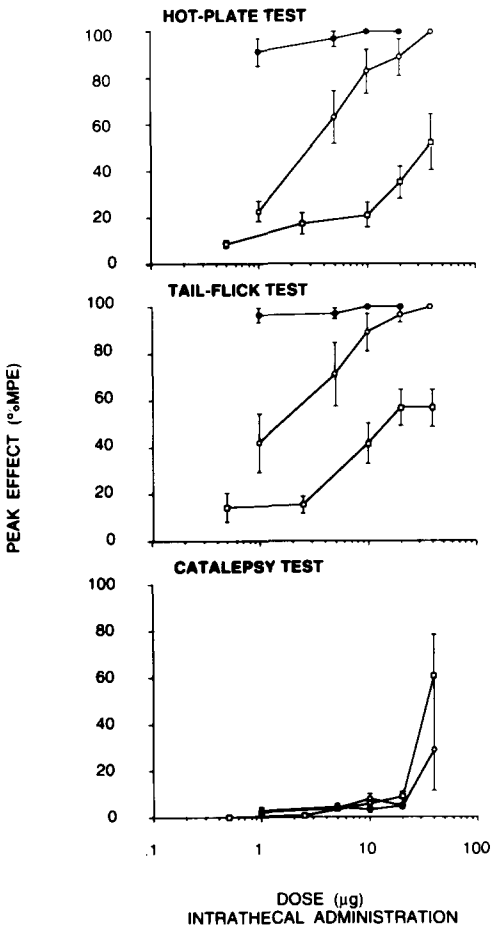

Fig. 2. The potentiating effect of intrathecal (i.t.) injection of a submaximal dose of midazolam (20 μg) on morphine-induced analgesia in rats. Dose-response curves are shown for antinociceptive effects in the hot-plate and tail-flick tests and for the incidence of catalepsy, after i.t. administration of the following in rats (five to seven per dose): morphine (○), midazolam (□) and a combination of morphine and 20 μg of midazolam (●) in rats. Antinociceptive effect is expressed as the peak percentage (mean ± S.E.M.) of the maximum possible effect (%MPE). For i.t. administration of saline ($n = 4$), peak %MPE was 3.24 ± 1.39% in the hot-plate test, 8.93 ± 2.36% in the tail-flick test and 3.03 ± 0.58% in the catalepsy test (saline administration did not produce motor dysfunction).

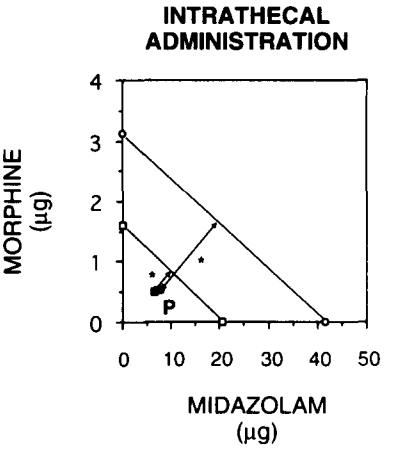

Fig. 3. Isobologram for intrathecal (i.t.) injections of equieffective doses of morphine, midazolam and a combination of morphine and midazolam in rats. Symbols on the y-axis represent the experimentally determined ED_{50} doses for morphine in the hot-plate test (○) and tail-flick test (□). Symbols on the x-axis follow the same scheme but refer to midazolam. The diagonal line is the locus of points representing theoretical additive doses. Black symbols at point P represent the combination of i.t. doses of midazolam and morphine that potentiated morphine-induced antinociception in the hot-plate (●) and tail-flick (■) tests.

almost the cutoff value, the peak values occurring 44.3 ± 5.7 min (hot-plate test) and 43.6 ± 4.6 min (tail-flick test) after drug injection (Fig. 1). As determined by ANOVA for repeated measures, for the drug effect, P was 0.0003 (hot-plate test) and 0.1 (tail-flick test); for the time effect, P was 0.0001 (both tests); and for the drug vs. time effect, P was 0.0018 (hot-plate test) and 0.0084 (tail-flick test).

Fig. 2 presents the dose-effect curves for the nociceptive tests and the test of catalepsy for midazolam, morphine and (midazolam + morphine) as %MPEs. Baseline nociceptive test results for rats undergoing i.t. injection ($n = 90$) remained stable over 30 min (baseline 1 and 2 values were 7.17 ± 0.21 s and 7.78 ± 0.22 s, respectively, for the hot-plate test; and 2.99 ± 0.03 s and 3.03 ± 0.03 s, respectively, for the tail-flick test). Baseline nociceptive tests did not differ between the groups.

I.t. injection of 1–40 μg of morphine increased the response latency on both nociceptive tests; the magnitude and duration of the increase related to dose (Fig. 2). In contrast, i.t. injection of 0.5–40 μg of midazolam produced only transient increases in hot-plate and tail-flick latencies (Fig. 2). The dose-response curve for peak effect (peak %MPE) of morphine was shifted to the left by the submaximal dose of midazolam (20 μg) in both nociceptive tests, indicating that a submaximal dose of midazolam potentiates antinociception produced by morphine. Data for AUC produced dose-response curves for morphine, midazolam and their combination that were similar to those produced by peak

%MPE values (data not shown). Also, the influence of a minimal effective dose of i.t. midazolam (2.5 μg) on i.t. morphine did not differ significantly in peak %MPE and AUC from that produced by saline (Table 1). Thus, when a minimal effective dose of midazolam (2.5 μg) and 1 μg of morphine were administered together i.t., midazolam potentiated the antinociceptive effect of morphine in both hot-plate and tail-flick tests (Table 1). This result indicates that minimal doses of each agonist produce a potentiating effect.

Isobolographic analysis showed that i.t. administration of equieffective doses of morphine and midazolam potentiated antinociception on the hot-plate and tail-flick tests. This is illustrated in Fig. 3 by the fact that a point on the isobole is located significantly to the left of the line representing theoretical additive doses.

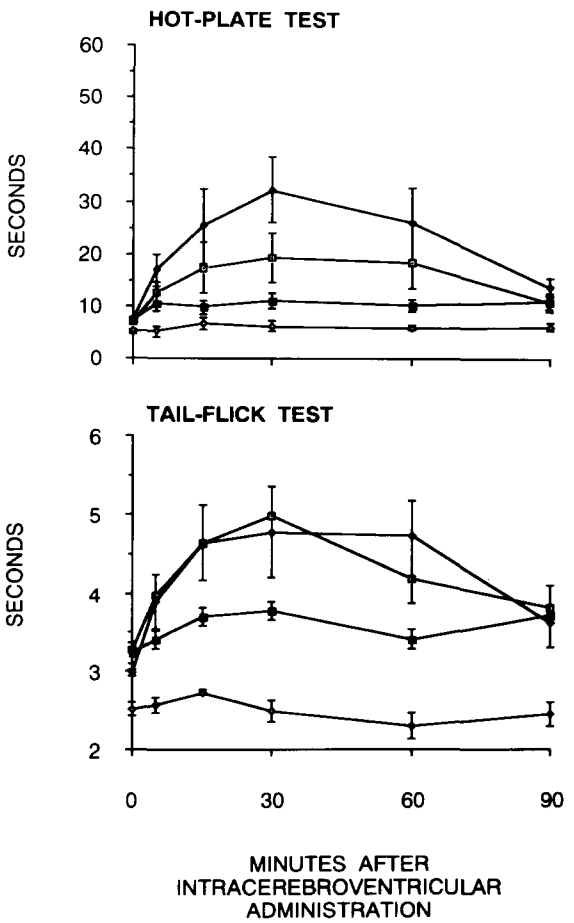

Fig. 4. Nociceptive response latencies (means ± S.E.M.) plotted against time for randomly chosen intracerebroventricular doses of morphine (2 μg, $n = 6$, ♦), midazolam (4 μg, $n = 9$, ■), a combination of the two (same doses, $n = 10$, □) and saline ($n = 4$, ◇) in rats. Co-administration of midazolam and morphine slightly decreased response latencies. The cutoff time was 60 s for the hot-plate test and 6 s for the tail-flick test. As determined by analysis of variance with repeated measures, $^{*}P \leq 0.05$.

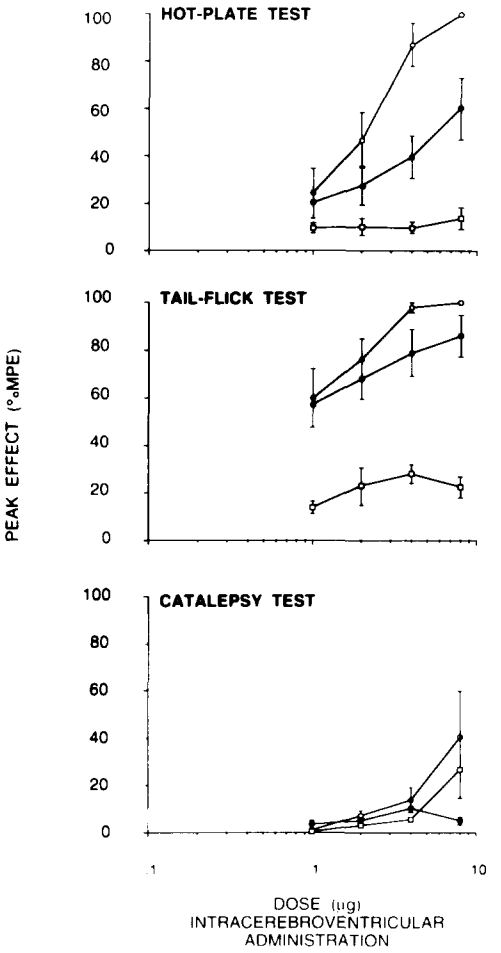

Fig. 5. The incidence of catalepsy and the inhibitory effect of intracerebroventricular (i.c.v.) administration of morphine (○), midazolam (□) and a combination of morphine and 4 μg of midazolam (●) on antinociception in rats (five to ten per dose). Antinociceptive effect is expressed as the peak percentage (mean ± S.E.M.) of the maximum possible effect in the hot-plate and tail-flick tests. For i.c.v. administration of saline ($n = 4$), peak %MPE was 4.35 ± 0.96% for the hot-plate test, 8.07 ± 1.84% for the tail-flick test and 3.24 ± 1.15% for the catalepsy test (saline administration did not produce motor dysfunction).

Motor function

Fig. 2 provides the incidence of catalepsy in rats given different i.t. doses of midazolam, morphine or both. For doses used to evaluate combinations of midazolam and morphine, the incidence was near zero (Fig. 2). As indicated, catalepsy was seen only at the highest doses of morphine (40 μg) or midazolam (40 μg) alone (Fig. 2). Quantitatively, three of six animals given 40 μg of i.t. morphine and four of five rats given 40 μg of i.t. midazolam had motor dysfunction with an index of 1.

3.2. Intracerebroventricular administration

Nociceptive response

Fig. 4 presents the changes in nociceptive response latencies after i.c.v. administration of randomly chosen

doses of morphine (2 μg), midazolam (4 μg) and a combination of the two (2 μg morphine + 4 μg midazolam). I.c.v. morphine (2 μg) slightly increased response latency, the peak value occurring 52.5 ± 10.8 min (hot-plate test) and 35.0 ± 8.4 min (tail-flick test) after drug injection. Co-administration of 4 μg of i.c.v. midazolam slightly reduced the response latency only in the hot-plate test, the peak value occurring 36.8 ± 5.5 min after injection. In the tail-flick test, 2 μg of i.c.v. midazolam did not significantly affect morphine analgesia; the peak value occurred at 44.3 ± 7.1 min. As determined by ANOVA for repeated measures, for the drug effect, P was 0.15 (hot-plate test) and 0.019 (tail-flick test); for the time effect, P was 0.0001 (both tests); and for the drug vs. time effect, P was 0.0005 (hot-plate test) and 0.0001 (tail-flick test). Baseline nociceptive test measurements for rats undergoing i.c.v. injection ($n = 97$) remained stable over 30 min and did not differ between groups (baseline 1 and 2 values were 6.8 ± 0.18 s and 7.16 ± 0.16 s, respectively, for the hot-plate test; and 3.03 ± 0.038 s and 3.098 ± 0.04 s, respectively, for the tail-flick test).

As indicated in Fig. 5, which shows peak effect (peak %MPE) plotted against dose, the i.c.v. administration of 1–8 μg of morphine alone increased hot-plate and tail-flick response latencies in a dose-dependent manner. I.c.v. injection of 1–8 μg of midazolam was not effective. The inhibitory effect of combining 4 μg of i.c.v. midazolam with morphine is demonstrated by a rightward shift of the morphine dose-response curve for the hot-plate test when compared with the curve representing the effects of morphine alone. In contrast, similar decrements in response latency were not observed in tail-flick assessments (Table 1). Because plotting of AUC data against dose produced basically the same information as did AUC vs. peak %MPE data, data are not shown separately.

Motor function

Only the highest i.c.v. doses of morphine (8 μg) or midazolam (8 μg) alone produced catalepsy (Fig. 5). Fifty percent of rats given i.c.v. injection of either morphine or midazolam at these dose levels had motor dysfunction with an index of 1 ($n = 6$). In striking contrast, high doses of midazolam (4 μg) and morphine (8 μg) given in combination did not produce catalepsy or motor dysfunction, indicating that midazolam also inhibits this motor effect of i.c.v. morphine by means of supraspinal actions.

4. Discussion

4.1. Effects in the spinal cord

We found evidence that midazolam potentiates and prolongs morphine-induced antinociception in hot-plate and tail-flick tests when both drugs are given i.t. at doses not affecting motor function. Synergism between two agents implies that an effect occurs in the presence of two agents that is greater than the sum of the effects produced by the same dose of those agents given separately. The fact that 2.5 μg of midazolam had no effect when given alone but produced significant increases in the presence of morphine supports the proposition that non-linear synergism occurs between morphine and midazolam. Additionally, isobolographic analysis (Tallarida et al., 1989) confirms the potentiating antinociceptive effect of the morphine-midazolam interaction at the spinal level.

Our results support previous observations that doses of midazolam having by themselves no effect on nociceptive assessments can potentiate and prolong morphine-induced antinociception, indicating the occurrence of a non-linear interaction (Moreau and Pieri, 1988; Yanez et al., 1990). Consistent with pharmacologic characteristics of the two classes of agents, previous work has demonstrated that synergism disappears when the opioid antagonist naloxone and the benzodiazepine inverse agonist Ro 19-4603 are administered (Moreau and Pieri, 1988; Yanez et al., 1990; Rattan et al., 1991). This result suggests that the presence of an opioid receptor was needed for the manifestation of synergism. Moreover, benzodiazepines are known to enhance the efficacy of GABA at $GABA_A$ sites (Haefely and Polc, 1986; Möhler and Richards, 1988; Haefely, 1989). Morphine can increase the amount of GABA and the activity of glutamate decarboxylase in dorsal parts of the spinal dorsal horn in rats (Kuriyama and Yoneda, 1978). The activity of the benzodiazepine inverse agonist on morphine-induced antinociception (Moreau and Pieri, 1988) supports the hypothesis that such a mechanism applies in our study. Previous work with i.t. muscimol has shown modest antinociceptive actions (Yamamoto and Yaksh, 1991). All the physiologic interactions of $GABA_A$ and μ-opioid sites advocate for this observed role of midazolam and morphine, and such a synergism between i.t. muscimol and morphine may be evident. Therefore, potentiation of morphine-induced antinociception by midazolam in the spinal cord may be the result of morphine stimulation of μ-receptors and, by means of GABAergic mechanisms, an overall inhibitory effect on spinal neural activity by benzodiazepines.

Several studies have reported that modulation of morphine-induced antinociception by midazolam may relate to the route of administration – systemic (Rosland and Hole, 1990a,b; Kissin et al., 1990), i.t. (Moreau and Pieri, 1988; Yanez et al., 1990) or i.c.v. (Mantegazza et al., 1982). These observations are consistent with our findings of a spinal potentiating effect and a supraspinal inhibitory effect, i.e., depending on the route of injection of these drugs (i.t. vs. i.c.v.), midazo-

lam would potentiate or inhibit morphine-induced antinociception. This result seems to be contrary to the report of Rattan et al. (1991), who, using only one route of administration (i.t.), indicated that enhancement or inhibition of morphine-induced antinociception by midazolam depended on the relative concentrations of the two drugs.

Discrepancies between our findings and those of Rattan and colleagues are as follows. First, even though both studies used similar dose ranges of 1–40 μg of i.t. midazolam, the periods of observation differed. Rattan et al. found that inhibition was more apparent between 2–6 h after injection of the drugs, whereas our period of observation had already ended by that time. However, it is unlikely that we would have observed any inhibiting effect after 2 h, because all doses (especially the highest) were reduced at this measurement point; at this time point, no inhibition could be observed with submaximal doses of midazolam. Second, if there had been an effect related to the relative concentration of the drugs administered via one route, we should have been able to find similar results with both routes of administration. Third, the difference in study results raises the question as to whether a late mild systemic drug effect would have activated mechanisms in the brain. Therefore, the late 'antagonistic effect' seen by Rattan et al. with one route of administration may be attributable to an influence of higher cerebral centers. Thus, at the spinal level, inhibition could be seen after the time interval in question. Fourth, incorporating a wide range of doses in our experimental design, we found that low (minimal effective) doses to high (submaximal) doses of midazolam potentiated morphine action at the spinal level, and that an antagonistic effect could only be seen at the supraspinal level.

4.2. Effects in the brain

We found that midazolam inhibited morphine-induced antinociception only in the hot-plate test and only when both drugs were injected supraspinally. Our results are consistent with previous reports that i.c.v. injection of diazepam and midazolam reduces the antinociceptive effect of systemically administered morphine in tail-flick tests (Mantegazza et al., 1982). Mid-thoracic spinalization abolishes the inhibitory effect of midazolam on morphine-induced antinociception in mice, indicating that a benzodiazepine-mediated mechanism blocks the effect of opioids on nociceptive transmission at higher levels of the central nervous system (Rosland and Hole, 1990b). Microinjections of midazolam into the dorsal raphe-periaqueductal gray area antagonizes the analgesic effect of morphine (Mantegazza et al., 1982). Consistent with these observations obtained with midazolam is the fact that the microinjection of muscimol, a $GABA_A$ receptor agonist, into the periaqueductal grey area (Zambotti et al., 1982) and cerebral ventricle (Mantegazza et al., 1979a), as well as the i.c.v. injection of GABA uptake inhibitors (nipecotic acid and guvacine) (Mantegazza et al., 1979b) and GABA itself (Yamamoto et al., 1981), inhibit morphine-induced antinociception in tail-flick tests. It should be noted that muscimol inhibits analgesia induced by i.c.v. but not i.t. morphine, indicating that the $GABA_A$ receptor is involved in modulating supraspinal actions of opioid receptor occupancy (Ding et al., 1990). Therefore, benzodiazepines may modulate the antinociceptive effect of opioids in the brain by means of physiologic mechanisms that are distinct from the effects of benzodiazepines in the spinal cord.

4.3. Influence on motor function

We took every precaution to select doses of drugs that would minimize possible motor function contamination and thus allow unambiguous conclusions. An increase in hot-plate and tail-flick latencies does not necessarily imply antinociception when accompanied by muscle flaccidity, catalepsy or motor incoordination (Hammond, 1989), and decreased motor function may have been interpreted as antinociception in some previous reports. To avoid this possible misinterpretation, behavioral measures of nociception, such as tail-flick and hot-plate tests, must be performed in parallel with evaluation of motor function (e.g., ratings of global motor function and catalepsy). However, a normal performance in these tests may not exclude some degree of motor dysfunction or mild sedation that may influence the response latency in the hot-plate test. In addition, the failure of midazolam to alter morphine-induced effects on motor function further emphasizes that the interaction of midazolam and morphine on antinociception is not a general property of opiate receptor function.

An important issue in interpreting the spinal and supraspinal interactions relates to anatomic localization of locally administered drugs. We observed that significant redistribution of injectate did not occur. Thus, our results probably were not attributable to direct actions on receptors in the spinal cord after i.c.v. injection, or in the brain after i.t. administration. As indicated, the administration of 0.9% sodium chloride had no significant antinociceptive or motor effects by itself, and our control parameters were comparable to each other. Furthermore, the antinociceptive effect was dose-dependent, whereas the volume remained constant. Another possible source of error of our results might be the tail-flick temperature that was not measured in this study. The tail-flick test may be particularly sensitive to changes in tail skin temperature (Hole and Tjølsen, 1993). However, it seems not likely that possible temperature changes induced by

the drugs would entirely explain the rather great changes in tail-flick latencies observed.

Our study design allowed us to administer, in the same set of experiments, both agents to both potential neuraxial sites of action. This advantage avoids the complications of having to compare i.t. results with i.c.v. results from different laboratories in order to draw conclusions. Second, this approach allowed us to separate clearly the influence of midazolam on the antinociceptive effect of morphine in the spinal cord from that in the brain. Third, clear delineation of the differing influence of i.t. vs. i.c.v. midazolam on morphine-induced antinociception provides a tool to examine which site of action – supraspinal or spinal – is more important in mediating morphine effects in tail-flick and hot-plate tests.

4.4. Conclusion

We have examined the effect of midazolam on morphine-induced antinociception after spinal or supraspinal administration in rats, with special emphasis on the concurrent evaluation of motor function. Midazolam potentiated and prolonged the spinal antinociceptive actions of morphine. On the other hand, when both drugs were administered at the supraspinal level, midazolam decreased the antinociceptive potency and duration of morphine. This differing effect of midazolam on morphine-induced antinociception suggests that different mechanisms are involved in the spinal cord and in the brain.

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