Pharmacology Biochemistry & Behavior, Vol. 37, pp. 505–509. © Pergamon Press plc, 1990. Printed in the U.S.A. 0091-3057/90 $3.00 + .00

Benzodiazepine-Induced Antagonism of Opioid Antinociception May Be Abolished by Spinalization or Blockade of the Benzodiazepine Receptor

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Received 13 March 1990

ROSLAND, J. H. AND K. HOLE. *Benzodiazepine-induced antagonism of opioid antinociception may be abolished by spinalization or blockade of the benzodiazepine receptor.* PHARMACOL BIOCHEM BEHAV **37**(3) 505–509, 1990.—The mechanisms underlying benzodiazepine antagonism of opioid antinociception were studied using the tail flick test and the hot plate test in mice. Both single-dose and repeated diazepam treatment antagonized the antinociceptive effect of morphine. The specific benzodiazepine antagonist flumazenil completely reversed the antagonism between diazepam and morphine. Mid-thoracic spinalization also abolished the antagonism, indicating that the antagonism takes place at higher levels in the CNS. Neither diazepam nor midazolam showed any affinity for opioid mu or kappa receptors in membranes prepared from mouse forebrain. Taken together with the results of other studies of interactions between GABAergic drugs and opioids, the results indicate that a benzodiazepine receptor-mediated mechanism at higher levels in the CNS, possibly in the brainstem, blocks the effect of opioids on nociceptive transmission.

Diazepam Morphine Flumazenil Antagonism Spinalization Opioid binding Mice

DIAZEPAM and other benzodiazepines have been shown to interact with the antinociceptive effect of morphine and other opioid drugs (1, 5, 12, 14, 17). In studies using mice and rats, some investigators have found that benzodiazepines increase opioid antinociception, while others have demonstrated an attenuated antinociceptive effect. Investigators using high doses of benzodiazepines conclude that there is either an unchanged opioid effect or an increased effect (4,22), while investigators using lower doses seem to demonstrate antagonistic effects (1, 5, 13, 19). Local injections of benzodiazepines either into brainstem structures or intraventricularly have been found to attenuate the effects of opioids (12,26).

Systemic administration of benzodiazepines induces sedation and muscle relaxation, which may complicate the interpretation of the responses to nociceptive stimulation (17–19). The doses of benzodiazepines used, therefore, seem to be critical in the studies of the interactions. Both benzodiazepines and opioids bind to specific receptors in the brain, and induce quite different effects. There are, therefore, several possibilities for the mechanisms underlying the interactions. The pharmacokinetics may be changed, or there may be an interaction at the receptor level. Stimulating GABAergic neurotransmission by benzodiazepines may modulate opioid transmission and vice versa (10).

In a previous study, we did not find any change in brain, spinal cord or serum concentrations of morphine in mice given diazepam, indicating no pharmacokinetic changes (18). In the present study, we have investigated the effect of spinalization as well as of repeated benzodiazepine treatment on the interaction. Derivatives of benzodiazepines have been shown to bind to opioid receptors (20), and we therefore also studied the binding of benzodiazepines to opioid receptors. The ability of a specific benzodiazepine receptor antagonist to block the benzodiazepine effect was also studied.

METHOD

Animals

Male albino mice (Bom:NMRI) weighing 25–35 g were used in the experiments. The animals were housed in colony cages (16 mice in each) with free access to food and water prior to the experiments. They were maintained in climate- and light-controlled rooms (22–23°C, 12/12 hr dark/light cycle with lights on at 7 a.m.) for at least two weeks prior to the experiments. Testing took place between 9 a.m. and noon. The animals were brought to the test room the day before testing, and were thus adapted to the testing environment for at least 18 hr. Two hr before testing, the animals were placed individually in standard macrolone cages ($30 \times 12 \times 13$ cm). Separate groups of animals were used for the different tests, except for the rotarod test, which was performed on animals tested in the tail flick test 5 min earlier.

Surgical Procedure

The animals were anesthetized with a combination of hypnorm (fentanyl 0.4 mg/kg + fluanison 12.5 mg/kg) and midazo-

lam 6.25 mg/kg. The 7th and 8th thoracic spine were localized, and a laminectomy was performed using a dental burr. The spinal cord was exposed and gently transected with a surgical knife. Complete transection was ensured by removing a slice of 1 mm of the cord. After proper haemostasis, the wound was closed, and the mice were given at least 7 days of recovery. The spinalized mice were tested in the tail flick test, and animals with no tail flick within 6 sec were rejected.

Drugs and Administration Route

Commercially available solutions of diazepam (Valium, Roche), midazolam (Dormicum, Roche), and morphine hydrochloride (NAF laboratoriene A/S) were used. Flumazenil (Ro 15-1788) was kindly donated by F. Hoffmann-La Roche & Co., Basel. For the behavioural studies, the drugs were diluted in 0.9% NaCl, to adjust the injected volume to 5 ml/kg. Diazepam, midazolam and flumazenil were injected intraperitoneally (IP), morphine was injected subcutaneously (SC) in the neck. Morphine, midazolam and diazepam were given 30 min prior to testing, flumazenil was given 20 min prior to testing. In all experiments an equal volume of 0.9% NaCl was used as control for the injections.

Test for Sensorimotor Performance

To evaluate the muscle relaxing and sedative effects of the benzodiazepines, the mice were tested on a rotarod (17). A horizontal wooden rod, 50 cm long and 4.2 cm in diameter, 100 cm above the floor, was driven by a motor. The speed of the rod was set to 2 cycles/min. With this speed, 90–100% of control mice balanced on the rod for 45 sec, which was the cut off time.

Nociceptive Assays

Tail flick test and tail skin temperature registration. Tail flick latency (6) was obtained using an IITC Inc. Model 33 Analgesiameter. Radiant heat was focused on a spot 1–2 cm from the tip of the tail, and the latency until the mouse flicked its tail was recorded. Beam intensity was adjusted to give a tail flick latency of 3–4 sec in control animals. The animals were restrained during trials by means of a Plexiglas cylinder 4 cm in diameter and 14 cm long.

The tail skin temperature was measured on the ventral surface, about 35 mm proximal to the spot where the beam was focused, by means of a copper-constantan thermocouple probe (0.2 mm copper-constantan wire, the junction measuring 0.4×0.8 mm).

Both the thermocouple and the tail flick apparatus were connected via a Biodata Microlink III interface unit to an IBM Personal Computer programmed to record the tail temperature and the tail flick latency simultaneously. The beam duration was controlled manually from the computer keyboard. The tail skin temperature was recorded at the moment when the tail flick occurred. This modification of the tail flick test has been described in detail previously (7,25).

Hot plate test. In the hot plate test (11), an IITC Inc. Model 35-D Analgesiameter was set to give a plate temperature of 55 ± 0.5°C. The animals were placed on the hot plate confined by a lidded perspex box with a compartment measuring 13.8×13.8 cm, and the latency to the first hind paw lick was recorded. If no hind paw lick occurred, the test was terminated after 30 sec. The animals were preadapted to the test apparatus by putting each animal in the perspex box on the cold plate for 1–2 min the day before testing.

Repeated Diazepam Treatment

Mice were given diazepam 1 mg/kg IP once a day for 8 days, control animals were given saline. On the eighth day, the animals were tested in the tail flick test and in the rotarod test. Before testing, diazepam-treated animals were given either morphine 3 mg/kg or saline together with the last diazepam dose, the saline-treated controls were given either morphine or saline with or without diazepam as shown in Fig. 3.

Opioid Receptor Binding

The mice were killed by decapitation, and the brain and spinal cord were quickly removed. The forebrain and the spinal cord were homogenized in 10 vol. of ice-cold 5 mM Tris-HCl buffer (pH = 7.4) with a teflon-glass homogenizer. The homogenate was centrifuged at 2500 rpm for 10 min, and the crude nuclear pellet was discarded. The supernatant was centrifuged twice at 18,000 rpm ($40,000 \times g$) for 20 min, the pellet washed with Tris-buffer, sonicated and suspended in 50 vol. of 5 mM Tris buffer, giving a final protein concentration of 0.3–0.4 mg/ml. Different concentrations of midazolam and diazepam were incubated for 30 min at 28°C with a fixed concentration of ^{3}H-DAGO or ^{3}H-U69593 (1 and 0.4 nM, respectively). Unspecific binding was determined in the presence of 5 μM of the cold ligand. The tubes were harvested in a Titertec cell harvester using GFB-filter. The filters were solved in 5 ml Filter-count scintillation fluid, and radiation was then counted in a LKB 1219 RackBeta Spectral Liquid Scintillation Counter. At each data point, 3–4 determinations were made, and all the experiments were done at least twice.

Serum Concentration of Diazepam

The measurement of the serum concentration of diazepam and its main metabolite N-demethyldiazepam was carried out using a modification of the gas chromatographic method of Berlin and co-workers (3). The internal standard (griseofulvin) was added after extraction with toluene/isoamylalcohol, and the extract was analyzed using a Hewlett-Packard Model 5730 gas chromatograph equipped with a ^{63}Ni electron capture detector and a model 3380 A recorder/integrator. All analyses were carried out in duplicate. Collection of blood samples was performed by puncture of the heart during combined pentobarbital and chloral hydrate anesthesia.

Statistical Analyses

Fisher's exact test was used to analyze the rotarod data. The tail flick and tail skin temperature data were analyzed by analysis of covariance (ANCOVA), and the hot plate data were analyzed by analysis of variance (ANOVA). Student's *t*-test was used in order to compare pairs of means. Statistical significance was accepted at the 5% level ($p<0.05$).

RESULTS

Serum Concentrations of Diazepam and N-Demethyldiazepam

The serum concentrations of diazepam and its main metabolite N-demethyldiazepam measured 30 min after injection were not significantly different between animals receiving a single injection and those receiving repeated injections for 8 days (Fig. 1).

Sensorimotor Performance

The performance on the rotarod was significantly impaired both after a single injection and after repeated injections of diazepam 1 mg/kg for 8 days ($p<0.05$, Fisher's exact test, Fig. 2).

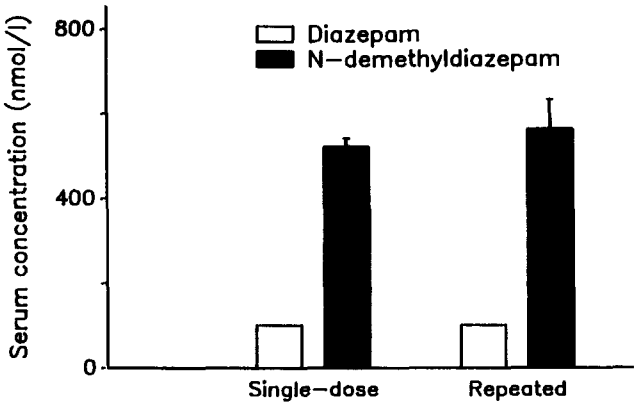

FIG. 1. Serum concentrations of diazepam and N-demethyldiazepam measured 30 min after a single dose of diazepam 1 mg/kg, or 30 min after the last injection of diazepam 1 mg/kg/day for 8 days. Mean ± S.E.M., n = 4–6 for each data point.

Diazepam-Morphine Interactions

Morphine 3 mg/kg induced a significant increase in tail flick latency [$t(37) = 4.999, p < 0.001$, Student's t-test]. The antinociceptive effect of this morphine dose was significantly reduced by diazepam 1 mg/kg both after a single injection and after repeated injections for 8 days [single-dose: $t(37) = 2.92$, $p < 0.01$, repeated: $t(41) = 3.42$, $p < 0.005$, Student's t-test, Fig. 3].

Effect of Thoracic Spinalization

Thoracal spinalization caused a considerable reduction of the effect of morphine in the tail flick test compared to intact controls (2). However, a significant dose-dependent antinociceptive effect could still be demonstrated after spinalization [$F(2,66) = 46.77$, $p < 0.001$, ANOVA, Fig. 4]. Diazepam in doses of up to 2 mg/kg did not influence the antinociceptive effect of morphine 4 or

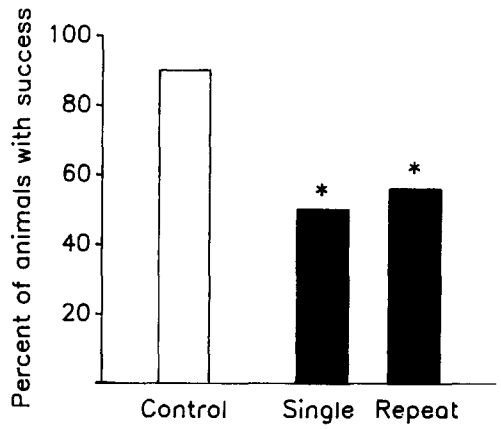

FIG. 2. Rotarod performance in mice given either diazepam 1 mg/kg as a single dose, or diazepam 1 mg/kg/day for 8 days, compared to control animals given saline. Results are expressed as the percentage of animals that succeeded to stay on the rod for 45 sec. Single = single-dose diazepam, Repeat = diazepam 1 mg/kg/day for 8 days. The animals were tested 30 min after drug injection, n = 16–18 for each dose level. Statistically significant differences compared to controls are indicated by $*p < 0.05$ (Fisher's exact test).

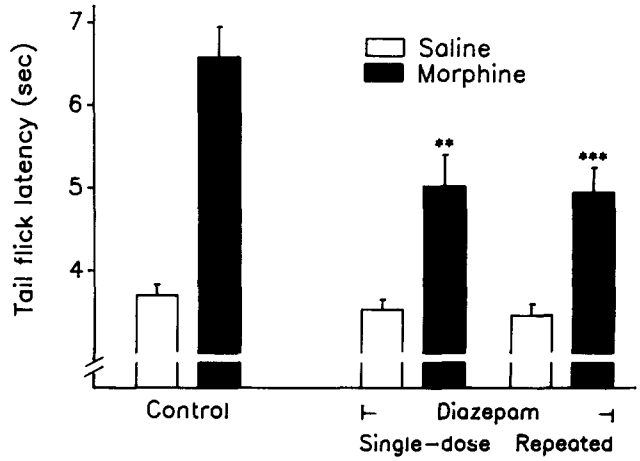

FIG. 3. The effect of single-dose or repeated administration for 8 days of diazepam 1 mg/kg/day on the tail flick latency in animals given saline or morphine 3 mg/kg. Mean ± S.E.M., n = 17–23 for each data point. Animals given single-dose or repeated diazepam treatment combined with morphine were compared to controls given morphine alone. Statistically significant differences are indicated by $**p < 0.01$ or $***p < 0.005$ (Student's t-test).

8 mg/kg in spinalized animals. Analysis of covariance (diazepam 2 mg/kg) indicated a significant covariance between tail flick latency and tail skin temperature, $F(1,66) = 10.71$, $p < 0.005$. No main effect of diazepam or interaction between diazepam and morphine was demonstrated [diazepam: $F(1,66) = 0.28$, $p > 0.6$, diazepam × morphine: $F(2,66) = 0.47$, $p > 0.6$, ANCOVA, Fig. 4].

Binding of Benzodiazepines to Opioid Receptors

Six concentrations of diazepam and midazolam ranging from 10 nM–5 μM were incubated with ^{3}H-DAGO (1 nM) or ^{3}H-U69593 (0.4 nM). No significant inhibition of opioid binding could be demonstrated with either drug (ANOVA, data not shown).

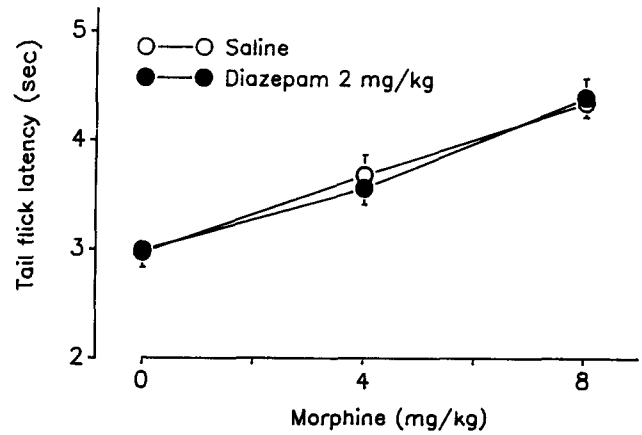

FIG. 4. The effect of diazepam 2 mg/kg compared to saline-treated controls on the dose-response to morphine in the tail flick test in spinalized animals. Mean ± S.E.M., n = 13–15 for each data point. Morphine alone induced a significant increase in tail-flick latency (4 mg/kg: $p < 0.01$, 8 mg/kg $p < 0.001$, Student's t-test subsequent to ANCOVA). This effect was not influenced by diazepam ($p > 0.6$, ANCOVA).

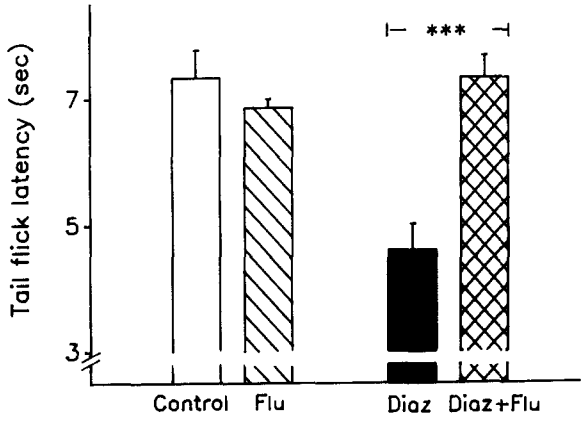

FIG. 5. The effect of flumazenil 10 mg/kg on the morphine antagonistic effect of diazepam 2 mg/kg in the tail flick test. All animals were given morphine 3 mg/kg, and the effect of diazepam, flumazenil or the combination of diazepam and flumazenil was studied. Control = morphine alone, Diaz = diazepam + morphine, Flu = flumazenil + morphine, Diaz + Flu = diazepam + flumazenil + morphine. Mean ± S.E.M., n = 8 for each data point. Statistical significant difference between the "Diaz" group and the "Diaz+Flu" group is indicated by ***$p<0.001$, Student's t-test.

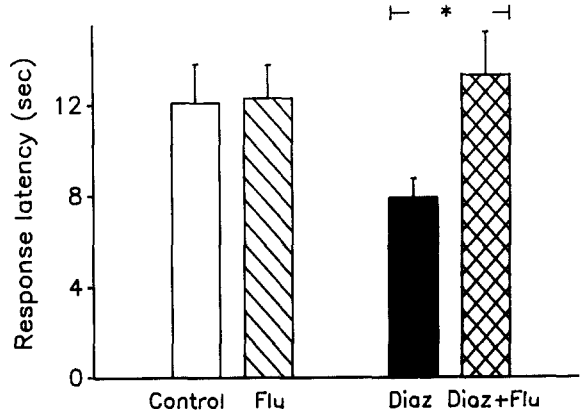

FIG. 6. The effect of flumazenil 10 mg/kg on the morphine antagonistic effect of diazepam 1 mg/kg in the hot plate test. All animals were given morphine 3 mg/kg, and the effect of diazepam, flumazenil or the combination of diazepam and flumazenil was studied. Mean ± S.E.M., n = 8 for each data point. Legends as for Fig. 5. Statistically significant difference between the "Diaz" group and the "Diaz+Flu" group is indicated by *$p<0.05$, Student's t-test.

Neither did the diazepam metabolite N-demethyldiazepam (100 nM–10 μM) induce any significant effect on μ-receptor binding (ANOVA, data not shown).

The Effect of Flumazenil on Diazepam-Morphine Interactions

Morphine 3 mg/kg almost doubled the tail flick latency and caused a 50% increase in response latency on the hot plate compared to saline-treated controls ($p<0.001$ and $p<0.05$, respectively, Student's t-test). This effect was significantly reduced by diazepam (2 mg/kg in the tail flick test, 1 mg/kg in the hot plate test). Flumazenil 10 mg/kg given 20 min before testing completely abolished the morphine antagonizing effect of diazepam in both tests (Figs. 5 and 6). Thus, the antagonizing effect of diazepam on morphine antinociception could be prevented by blocking the benzodiazepine receptor. Flumazenil itself did not influence the effect of morphine in either test. Statistical evaluation of the data indicated a significant reversal of the diazepam effect in both tests [tail flick test: $t(14)=7.17$, $p<0.001$, hot plate test: $t(18)=2.59$, $p<0.02$, Student's t-test].

DISCUSSION

The main results in this study were that the specific benzodiazepine antagonist flumazenil completely blocked the antagonistic effect of diazepam on morphine antinociception. Spinalization at mid-thoracic level completely abolished the interaction between diazepam and morphine in the tail flick test, indicating that the antagonism requires communication between the spinal cord and higher brain centers. Neither midazolam nor diazepam were able to inhibit mu or kappa opioid receptor binding in membrane preparations from mouse forebrain. Eight days of repeated diazepam treatment did not significantly change the interaction between diazepam and morphine in the tail flick test, and the performance on the rotarod was also unchanged, indicating no tolerance development with this experimental design. The serum concentration measurements of diazepam and its main metabolite N-demethyldiazepam indicated no accumulation of diazepam metabolites or other pharmacokinetic changes after repeated diazepam treatment.

Diazepam and other benzodiazepines bind to benzodiazepine receptors in the brain, and thereby facilitate GABAergic neurotransmission (16). The mechanisms for the modulation of nociception by benzodiazepines and GABAergic systems are still debated (10,21). It has been demonstrated that synthetic benzodiazepine derivatives may bind to opioid receptors in the brain (20), and it seems possible therefore that benzodiazepines also might bind to these receptors. However, we were not able to demonstrate any affinity for opioid mu or kappa receptors in mouse forebrain of diazepam or midazolam using concentrations of up to 5 μM, which is far beyond the concentrations that were measured in serum. In preliminary studies we found that using the same experimental procedure, morphine 10 nM caused a nearly complete inhibition of ^{3}H-DAGO binding. The main metabolite of diazepam, N-demethyldiazepam, did also not show any affinity for the mu receptor, indicating that binding to the opioid receptors is not the mechanism by which benzodiazepines inhibit the effect of opioids. The specific benzodiazepine receptor antagonist, flumazenil, completely blocked the morphine antagonistic effect of diazepam both in the hot plate test and the tail flick test, indicating that the interaction depends on an activation of benzodiazepine receptors. Zambotti *et al.* (26) have demonstrated similar effects of flumazenil in rats using intraventricular injection of the drugs. In mice, Palaoglu and Ayhan found that flumazenil partly reversed the diazepam-morphine antagonism, a complete reversal could only be demonstrated with the combined action of flumazenil and the chloride channel blocker picrotoxin (14).

Benzodiazepines are known to induce sensorimotor impairment (17,24). The interpretation of the results in tests of nociception may therefore be difficult. The hot plate test has shown to be more sensitive to these effects than the tail flick test (18,19), and we have found that the highest diazepam dose to give reliable results in this test was 1 mg/kg. In a previous study we have demonstrated a dose-dependent attenuation of several opioids using diazepam 0.5–2.0 mg/kg in the tail flick test and 0.2–1.0 mg/kg in the hot plate test (19). In the present study we have therefore used diazepam 1 mg/kg in the hot plate experiments and 1 or 2 mg/kg in the tail flick experiments. The chosen morphine dose (3 mg/kg) approximately doubled the tail flick latency, and caused

a 50% increase in response latency in the hot plate test. Similar responses of morphine in rodents have also been demonstrated by others (11,23).

Several 1,4-benzodiazepines seem to antagonize the effect of opioids in a similar manner as diazepam (5,19). However, the antagonism cannot be demonstrated in all types of nociceptive tests (18), indicating that either the level of integration of the response, or the type of stimulus could be critical for the interaction to take place. In the present study we did not find any antagonism in the spinalized mice, indicating that higher centers in the CNS have to be involved. It has been demonstrated that benzodiazepines injected into the periaqueductal gray matter may antagonize morphine antinociception in rats (12). Pan and Williams (15) found that opioids injected into nucleus raphe magnus decreased the activity of GABA-ergic neurons, suggesting that this could be one mechanism by which opioids modulate nociceptive inputs. It has also been demonstrated that GABA-agonists and GABA-antagonists injected into the brainstem may modify nociception measured by the tail flick test (10). All these results indicate that brainstem structures could be an important site for benzodiazepine-opioid interactions.

Chronic drug treatment has been shown to change the receptor sensitivity, for instance in serotonergic and opioid systems (8,27). Tolerance to some benzodiazepine effects has been demonstrated after chronic treatment, however, almost no tolerance to the muscle relaxing effects have been observed (9,24). In our experimental setting, no tolerance either to the muscle relaxing effect or to the morphine antagonistic effect could be demonstrated after 8 days following diazepam 1 mg/kg/day. It could be argued that the chosen dosing regimen was too low to demonstrate a possible tolerance development, and the tail flick experiment was therefore repeated using diazepam 2 mg/kg/day, giving a similar result.

In conclusion, diazepam-induced inhibition of morphine antinociception in mice may be reversed by flumazenil, indicating that activation of benzodiazepine receptors is involved. Spinalization completely abolished the diazepam effect, indicating that the interaction takes place at higher levels in the CNS, possibly in the brainstem. The morphine-diazepam antagonism was unchanged after 8 days of diazepam treatment. No influence of benzodiazepines on opioid receptor binding could be demonstrated. These results therefore indicate that the morphine antagonistic effect of diazepam takes place at a supraspinal level and is not linked to opioid receptors.

ACKNOWLEDGEMENTS

J. H. Rosland is a fellow of the Norwegian Cancer Society-Den Norske Kreftforening. We would like to thank Mr. A. Madsen for excellent technical assistance, and Dr. P. M. Ueland and Mr. G. Wallem for performing the analyses of diazepam and N-demethyldiazepam.

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