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Supraspinal Flumazenil Inhibits the Antianalgesic Action of Spinal Dynorphin A (1–17)

JODIE J. RADY, BLYTHE B. HOLMES AND JAMES M. FUJIMOTO

Research Service and Department of Pharmacology and Toxicology, Veterans Affairs Medical Center, Milwuakee, WI 53295

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RADY, J. J., B. B. HOLMES AND J. M. FUJIMOTO. *Supraspinal flumazenil inhibits the antianalgesic action of spinal dynorphin A (1–17)*. PHARMACOL BIOCHEM BEHAV **60**(1) 245–254, 1998.—DynorphinA (Dyn) administered intrathecally or released spinally in mice produces antianalgesia, that is, antagonizes morphine analgesia (tail-flick test). Spinal transection eliminates this Dyn antianalgesia. Present results in mice show that intracerebroventricular administration of flumazenil, a benzodiazepine receptor antagonist, also eliminated the antianalgesic action of Dyn; flumazenil in the brain eliminated the suppressant effect of intrathecal Dyn on intrathecal and intracerebroventricular morphine-induced antinociception. Intracerebroventricular clonidine, naloxone, and norbinaltorphimine release spinal Dyn. The latent antinociceptive actions of these compounds were uncovered by intracerebroventricular flumazenil. Thus, Dyn, given intrathecally or released spinally, activates a pathway that is inhibited by intracerebroventricular flumazenil. Dyn antianalgesia is not significantly altered by intracerebroventricular administration of bicuculline and picrotoxin, suggesting that activation of the gamma-aminobutyric acid receptor has little if any involvement in the antianalgesic action of Dyn. The antagonistic effect of Dyn seems to be mimicked by benzodiazepine agonists. Furthermore, administration of a benzodiazepine receptor inverse agonist (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) inhibited Dyn antianalgesia as did flumazenil. Thus, flumazenil, through a benzodiazepine antagonist or inverse agonist action, interrupts, as does spinal transection, the neuronal circuit (cord/brain/cord) necessary for the antianalgesic action of spinal Dyn. Because Dyn antianalgesia is an indirect action, activation of the neuronal circuit must lead to the release of a direct-acting antianalgesic mediator in the spinal cord. © 1998 Elsevier Science Inc.

Spinal dynorphin A Antianalgesia Flumazenil supraspinal Benzodiazepine receptor Antinociception Naloxone Norbinaltorphimine Clonidine Morphine Physostigmine

OUR previous studies demonstrate that dynorphin A (1–17), Dyn, administered intrathecally (IT) in mice antagonizes the antinociceptive action (in the tail-flick test) produced by a variety of analgesic agents (such as physostigmine and certain opioids) given intracerebroventricularly (ICV) (14–17) and morphine given IT (13,37,38). This action of Dyn is referred to as antianalgesia and is eliminated by IT administration of opioid antagonists (naloxone, β-funaltrexamine, norbinaltorphimine) and dynorphin antiserum (13–15,17,22,37).

Dyn can also produce an antianalgesic effect when released endogenously in the spinal cord following ICV administration of clonidine (an α_2-adrenergic agonist), physostigmine (a cholinesterase inhibitor), and midazolam (a benzodiazepine agonist). For example, clonidine given ICV releases spinal Dyn to antagonize the antinociceptive action of IT morphine (13,15). Administration of clonidine ICV produces minimal amounts of antinociception by itself because the presence of Dyn obscures the antinociceptive action. Attenuation of Dyn antianalgesia by IT administration of naloxone, norbinaltorphimine, and dynorphin antiserum enhances the antinociceptive action of ICV clonidine. In contrast to the effect of IT naloxone and norbinaltorphimine to attenuate the antianalgesic action of Dyn, ICV administration of these and certain other opiate antagonists results in release of spinal Dyn (1,21). Thus, ICV naloxone and norbinaltorphimine antagonize IT morphine-induced antinociception through the Dyn antianalgesic mech-

Requests for reprints should be addressed to James M. Fujimoto, Ph.D., Research Service-151, VA Medical Center Milwaukee, WI 53295.

anism. This action of ICV naloxone and norbinaltorphimine can be eliminated by treatments that attenuate Dyn action (21), such as naloxone, norbinaltorphimine, or Dyn antiserum administered IT.

Administration of midazolam (a benzodiazepine agonist) ICV also antagonizes IT morphine antinociception in mice by release of spinal Dyn (37). Flumazenil administered ICV inhibits this antagonistic action of midazolam. Even though the action of flumazenil is attributed to interaction with midazolam at the benzodiazepine receptor in the brain (31), an alternate possibility is that the flumazenil might be antagonizing the effect of spinal Dyn. The main purpose of the present study was to support this alternative argument by demonstrating that ICV flumazenil inhibits the antianalgesic action of spinal Dyn. ICV midazolam is not a good choice to test this proposal because it not only releases spinal Dyn but also acts on the benzodiazepine receptor. As described below, choosing drugs that release spinal Dyn but do not act on benzodiazepine receptors eliminates this concern. The alternative that flumazenil acts in the brain to antagonize the effect of spinal Dyn becomes a possibility because the spinal antianalgesic action of Dyn requires a pathway from the spinal cord to the brain. The antinociceptive action of IT morphine in the tail-flick test remains intact after spinal transection; however, the antianalgesic action of IT Dyn is abolished (51). Furthermore, a brief report (5) indicates that an antinociceptive action of naloxone is uncovered by the systemic administration of flumazenil. Because ICV naloxone has antianalgesic activity through the release of spinal Dyn (21), inhibition of the action of Dyn at the spinal site with opiate antagonists and Dyn antiserum uncovers the antinociceptive action of naloxone (unpublished data). In the present study, we propose that ICV flumazenil also inhibits the action of spinal Dyn to uncover the antinociceptive action of ICV naloxone. Other agents that release spinal Dyn following ICV administration are included to further demonstrate the ability of ICV flumazenil to enhance antinociceptive activity by inhibiting the antianalgesic action of endogenously released Dyn. Possible mechanisms for this action of flumazenil examined in this study include benzodiazepine receptor antagonist or inverse agonist activity and relationship to gamma-aminobutyric acid (GABA) receptors.

METHOD

Animals, Antinociceptive Test, and Statistical Analyses

All experiments used male ICR and CD-1 mice, weighing 25–35 g, from Sasco, Inc. (Omaha, NE) and Charles Rivers (Wilmington, MA), respectively (for all practical purposes these mice are the same). Mice were housed five per cage in a temperature-controlled room with a 12 L:12 D cycle, with lights on at 0700 h. Food and water were available ad lib. Each animal was used only once.

Antinociception was determined by the radiant heat tail-flick test described by D'Amour and Smith (9). The dorsal surface of the tail was exposed to a beam of light with the intensity set to provide predrug latencies (the average of two tail-flick latencies taken approximately 5 min apart) of 2–4 s. A 10-s cutoff time was used as the maximum antinociceptive response to prevent tail damage. Antinociception is reported as percent maximum possible effect (% MPE) as calculated according to the formula (10):

$$\%\ \text{MPE} = \frac{(\text{post drug latency} - \text{predrug latency})\,100}{10 - \text{predrug latency}}.$$

The data are presented as mean % MPE ± SEM for groups of 8–10 mice. When only two experimental groups were involved (as in the experiments for determination of the duration of action of flumazenil and its enhancement of physostigmine, naloxone, or norbinaltorphimine antinociception) the mean % MPE values were compared for a statistically significant difference using Student's *t*-test. For the experiment to determine the effect of various doses of flumazenil on clonidine action, one-way analysis of variance (ANOVA) was performed followed by Dunnett's test for post hoc comparisons of the mean % MPE of one control group to that of the flumazenil-treated groups. Experiments that required comparison of all experimental groups to one another were analyzed by ANOVA followed by Newman–Keuls' test for post hoc evaluation of individual group differences. In all tests, significant differences were indicated by $p < 0.05$ (45). In one experiment, dose–response data for intraperitoneal (IP) clonidine in the presence of saline or flumazenil, given IP, were fit to straight lines and the slopes and ED_{50} values were determined and compared using the method of Litchfield and Wilcoxon (30) as described previously by Dewey et al. (10).

Drug Source and Administration

Physostigmine salicylate, clonidine hydrochloride, yohimbine hydrochloride, and picrotoxin were obtained from Sigma (St. Louis, MO). Morphine sulfate was obtained from Mallinckrodt (St. Louis, MO). [D-Pen^2-D-Pen^5]enkephalin (DPDPE) was obtained from Bachem, Inc. (Torrance, CA). Dyn was obtained from Peninsula Laboratories (Belmont, CA). Naloxone hydrochloride was obtained from Dupont Pharmaceuticals (Garden City, NY). Norbinaltorphimine dihydrochloride, (+)-bicuculline, and DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) were obtained from Research Biochemicals International (Natick, MA). Flumazenil (Ro15-1788) was a gift from Hoffmann–La Roche (Nutley, NJ). Doses used refer to the drugs in the form stated above. All drugs, except for DPDPE and Dyn were dissolved in 0.9% saline. The peptides were dissolved in 0.01% Triton X-100 solution in 0.9% saline. A few drops of 0.1 M hydrochloric acid and slight heating was needed to fully dissolve bicuculline and flumazenil.

Drug or drug vehicle solutions were administered free hand into a lateral ventricle (ICV) in a 4 μl volume using a 22-gauge stainless steel needle attached to a 25 μl syringe according to the method of Haley and McCormick (20) under light halothane anesthesia or into the intrathecal space between the fifth and sixth lumbar vertebrae (IT) in a 5 μl volume using a 30-gauge needle attached to a 50 μl syringe according to the method of Hylden and Wilcox (23). In one experiment where the intraperitoneal (IP) route was used, drug solutions were administered in a volume of 0.1 ml/10 g b.wt. The dose and time of drug administration for peak effects were taken from previous studies (13,16,17,41,48) or determined in preliminary experiments. These parameters are stated in the figures for each experiment. The treatment time stated is the time between drug administration and the tail-flick test. In general, the times for administration of solutions ICV was 10 min and IT was 5 min (not mentioned further in text). One exception was in the experiment where the duration of action of ICV flumazenil was determined. Because a number of different combinations of drug administration parameters were used, the general approaches followed by specific details are given below. All experiments were performed in compliance with the Institutional Animal Care and Use Committee (Animal Studies Subcommittee).

Evaluation of the effect of ICV flumazenil on the antianalgesic action of IT Dyn

In one of the experiments, the ability of Dyn given IT to decrease the antinociceptive action of opioids given ICV or IT compared to the opioid treatment alone was examined. Superimposed on this protocol, the ability of ICV flumazenil to modify the antianalgesic action of Dyn was evaluated. The treatments for Set A are as follows: group 1—ICV morphine (4 μg) + IT saline (5 μl); group 2—ICV morphine (4 μg) + IT Dyn (10 pg); group 3—ICV morphine (4 μg) + ICV flumazenil (0.5 μg) + IT Dyn (10 pg) with the morphine and the flumazenil administered in the same solution. In set B, DPDPE (10 μg) was used in place of the morphine ICV with other treatments remaining the same. In set C, IT morphine (1 μg) was used in place of the ICV morphine. For set D, the treatment was as in set C but flumazenil was given IT instead of ICV. These experiments involved multiple group comparisons with ANOVA followed by Newman–Keuls' test.

In the above experiments (A and C), a single dose of morphine was used ICV and IT. To characterize the interactions further, full dose–response curves for ICV and IT morphine were generated in mice treated with IT saline (5 μl), IT Dyn (two doses) or a combination of ICV flumazenil (0.5 μg) and IT Dyn (20 pg). These dose–response curves took on an inverted U-shaped appearance; therefore, the ED_{50} values were not determined.

To determine if flumazenil had antinociceptive activity following ICV administration 0.5, 1, and 5 μg of flumazenil were given ICV; saline (5 μl) was also administered for comparison. The mean % MPE values for the flumazenil groups and the saline group were compared to each other by ANOVA.

Evaluation of the Effect of ICV Flumazenil on the Antianalgesic Effect of Endogenously Released Spinal Dyn

Clonidine and physostigmine given ICV produce antinociception while simultaneously releasing spinal Dyn (13–15,17); attenuating the effect of this endogenous Dyn component enhances the analgesic actions of the clonidine and physostigmine. Naloxone (nonselective opioid receptor antagonist) and norbinaltorphimine (κ antagonist) administered ICV produce little overt antinociception in the tail-flick test because of the presence of a Dyn antianalgesic component and attenuation of this Dyn component uncovers latent analgesic actions (13,21). These agents were administered together with ICV flumazenil in a single dose paradigm. If flumazenil attenuates the Dyn antianalgesic component, then the antinociceptive action of the agents should be enhanced (clonidine and physostigmine) or become overt (naloxone, norbinaltorphimine).

Clonidine Experiments

A: clonidine, 3 μg, was administered ICV along with various doses of flumazenil ICV (given in the same solution as clonidine) to determine the dose–response relationship for the effect of flumazenil. These groups were compared using ANOVA followed by Dunnett's test to compare the groups treated with flumazenil to the group treated with only clonidine. B: the time course for flumazenil effects was studied by giving flumazenil (0.5 μg) or saline (4 μl) ICV at various times with ICV clonidine given at a fixed dose (3 μg) and time. The statistical evaluation involved comparisons between flumazenil and saline-treated groups at each treatment time using Student's *t*-test. C: the ability of flumazenil to enhance clonidine analgesia was extended to systemic drug administration by examining the effect following IP administration of both compounds. First, to determine the duration of action of flumazenil, clonidine was given IP at a fixed dose (0.1 mg/kg) and time (30 min) and saline (0.1 ml/10 g b.wt.) or a fixed dose of flumazenil (10 mg/kg) was given IP at various times before the tail-flick test (when the time for flumazenil administration was the same as that for clonidine the two drugs were administered in the same solution). Second, to derive dose–response curves, various doses of clonidine were given IP 30 min before the tail flick test along with IP saline (0.1 ml/10 g) or flumazenil (10 mg/kg) given 20 min before the tail flick test. The ED_{50} values for IP clonidine-induced antinociception were compared (as stated above) to assess the effect of the flumazenil treatment.

Physostigmine, Naloxone, and Norbinaltorphimine Experiments

The ability of ICV flumazenil to inhibit the antianalgesic action of endogenously released Dyn was further demonstrated by administering physostigmine (2 μg), naloxone (1 ng), or norbinaltorphimine (1 μg) alone or in the same solution as flumazenil (0.5 μg) ICV. The antinociceptive response of the flumazenil treated groups were compared to that of the groups not receiving flumazenil using Student's *t*-test.

Involvement of Supraspinal GABA Receptors and Benzodiazepine Receptor Inverse Agonist Actions in Dyn Antianalgesia

Because flumazenil is a benzodiazepine receptor antagonist and the benzodiazepine receptor is associated with the GABA receptor chloride ionophore [see review (36)], activation of the GABA receptor may also be involved in Dyn antianalgesia. Bicuculline, a competitive $GABA_A$ receptor antagonist (8,42), and picrotoxin, a noncompetitive chloride ion channel blocker (42), were given ICV (1 μg and 0.25 μg, respectively) in an attempt to determine whether they blocked the antianalgesic action of IT Dyn (10 pg) against ICV morphine (4 μg). The protocol was as in Set A above with the bicuculline and picrotoxin given in place of flumazenil. To determine if the antianalgesic action of Dyn involved the release of a benzodiazepine receptor inverse agonist, DMCM (0.1 and 1 μg) was given along with morphine (4 μg) ICV. These groups were analyzed statistically using ANOVA. To determine whether DMCM inhibits IT Dyn antianalgesia, a protocol similar to Set A was used with DMCM (0.1 μg) given ICV in place of the flumazenil.

RESULTS

The Effect of IT Dyn to Antagonize Morphine Is Attenuated by ICV Administration of Flumazenil

Figure 1 shows the antinociception produced by ICV and IT morphine (A and C, respectively) and ICV DPDPE (B). In each case, IT administration of Dyn decreased the antinociceptive effects as expected (15). ICV administration of flumazenil eliminated this antianalgesic action of IT Dyn (Fig. 1A–1C). Furthermore, Dyn administered IT (with ICV saline) or with ICV flumazenil did not produce an antinociceptive response (saline ICV + Dyn IT = 14.3 ± 3.7%; flumazenil ICV + Dyn IT = 10.2 ± 4.2%).

Administration of varied doses of flumazenil ICV demonstrated that flumazenil by itself had no significant antinocicep-

TABLE 1

TAIL-FLICK RESPONSE TO SALINE OR FLUMAZENIL GIVEN ICV IN THE TAIL-FLICK TEST

	% MPE ± SEM	
Dose (μg)	Set 1	Set 2
Saline	2.6 ± 0.9	9.5 ± 4.1
0.5	12.7 ± 5.9	13.2 ± 3.8
1	14.0 ± 6.4	16.0 ± 3.1
5	3.7 ± 1.8	20.6 ± 5.4

No significant differences were found using ANOVA, Set 1, $F_{3, 35} = 1.83$ and Set 2, $F_{3, 36} = 1.25$; ($p > 0.05$). There were 10 mice in each group except for 9 at 0.5 mg dose in set 1.

tive activity (Table 1). Doses ranging from 0.5 to 5 μg (shown later to enhance the antinociceptive action of certain agents) produced no antinociception. Thus, the effect of ICV flumazenil on IT Dyn action was not due to antinociceptive activity of flumazenil.

Antagonism of IT Dyn Action Is Not Obtained by IT Administration of Flumazenil

Flumazenil given IT (Fig. 1D) did not produce the same effect as when given ICV. The ability of IT Dyn to antagonize IT morphine-induced antinociception was not altered by administration of flumazenil IT. The dose of flumazenil given IT was equal to the effective ICV dose; therefore, the ability of ICV flumazenil to antagonize the antianalgesic action of IT Dyn could not have been due to diffusion of flumazenil from the brain down to the spinal cord.

Dose–Response Curves for the Antagonism of ICV and IT Morphine by IT Dyn and Attenuation of This Dyn Action by ICV Flumazenil

Figure 2 presents the dose–response curves for ICV and IT morphine (A and B, respectively) as modified by IT administration of two different doses of Dyn. Because of the inverted U-shaped dose–response curves for morphine, comparison of ED_{50} values for morphine in the presence and absence of IT Dyn was not possible. However, the antianalgesic effect of Dyn against morphine was noncompetitive in that larger doses of morphine did not overcome the effect of Dyn, and ef-

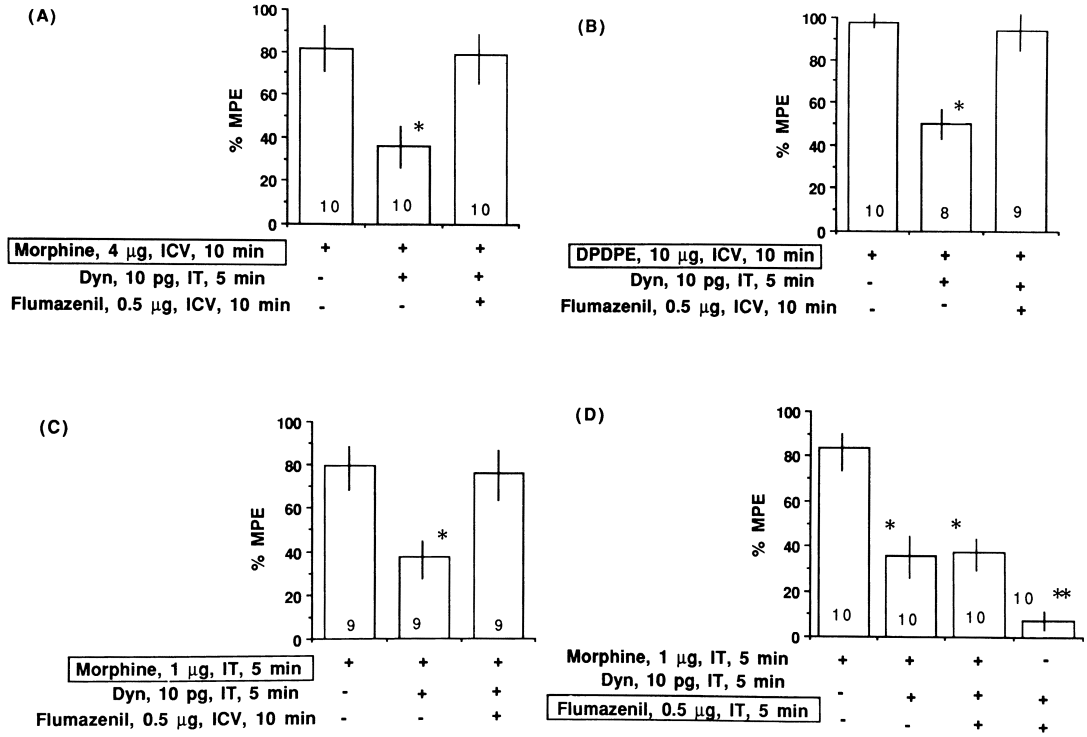

FIG. 1. (A) The antagonistic effect of IT Dyn against ICV morphine (group 1 vs. 2) was inhibited by ICV administration of flumazenil (group 2 vs. 3). *Indicates in A, B, and C that the group is significantly different from the other groups using ANOVA followed by Newman–Keuls' test; $p < 0.05$. In this and subsequent figures, a "+" and "−" under the bar indicates that the drug stated to the left or the appropriate vehicle, respectively, was given. The number in the bottom of the bar is the number of mice used for each group. (B) The antagonistic action of IT Dyn against ICV DPDPE (group 1 vs. 2) was attenuated by flumazenil given ICV (group 2 vs. 3). (C) Administration of flumazenil ICV eliminated the antagonistic action of Dyn against IT morphine (group 2 vs. 3). (D) Administration of flumazenil IT did not alter the antagonistic effect of Dyn against IT morphine-induced analgesia (group 2 vs. 3). Flumazenil and Dyn coadministered IT did not produce an antinociceptive response (group 4). **Indicates that the group is significantly different from all other groups and *indicates that the groups are significantly different from the other groups but not each other using ANOVA followed by Newman–Keuls' test; $p < 0.05$.

ficacy comparable to ICV and IT morphine alone were not attained in the presence of Dyn. For the more immediate purpose, the antianalgesic action of IT Dyn against ICV and IT morphine was inhibited by ICV administration of flumazenil. The responses for ICV and IT morphine (the latter at doses of 0.5 μg and above) were brought back to control values by ICV flumazenil administration (Fig. 2). However, at the 0.1 μg dose of morphine IT, the response was greater with the flumazenil treatment than that for morphine alone (see Discussion). The results indicated that ICV flumazenil inhibited the antianalgesic action of IT Dyn. The notable feature was that the antagonism of IT Dyn was indirect in that the Dyn and flumazenil were given at sites that were remote from each other.

Flumazenil Enhances Clonidine-Induced Antinociception

The next consideration was whether ICV flumazenil administration would inhibit the antianalgesic action of Dyn released endogenously in the spinal cord. Clonidine given ICV produced minimal antinociception (Fig. 3A). When a 0.5 μg or higher dose of flumazenil was administered together with clonidine ICV, an increase in antinociception was obtained. In-

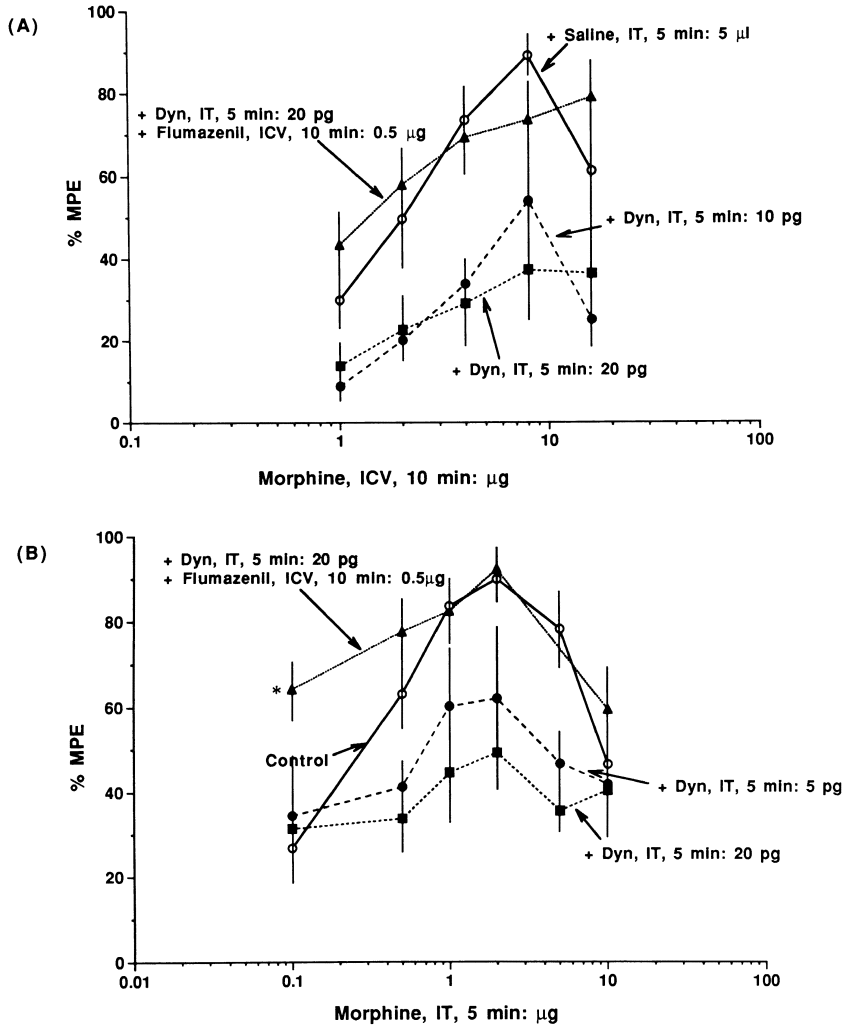

FIG. 2. (A) Morphine given ICV in the presence of IT saline produced an inverted U-shaped antinociceptive response (open circles). Administration of a 10 pg (filled circles) and 20 pg (filled squares) dose of Dyn IT decreased the antinociceptive response to ICV morphine. The effect of the 20 pg dose of Dyn was reversed by administration of flumazenil ICV (filled triangles). (B) Morphine given IT also produced an inverted U-shaped dose–response relationship (open circles). The antinociceptive response to IT morphine was attenuated by coadministration of 5 and 20 pg of Dyn (filled circles and filled squares, respectively). Again, the effect of Dyn (20 pg) was inhibited by giving flumazenil ICV (filled triangles). *Indicates significant difference, $p < 0.05$, from corresponding control mean (open circle) by Student's *t*-test.

creases in the flumazenil dose between 0.5 and 5 μg did not lead to a further increase in clonidine-induced antinociception.

Figure 3B shows the duration of action of the effect of flumazenil to increase clonidine-induced antinociception. Enhancement was evident when flumazenil was given at 10 min and still present at 15 h (900 min), but gone by 24 h (1440 min) before the tail-flick test. Thus, flumazenil had a long duration of action for increasing the antinociceptive response of clonidine.

Because the systemic routes of administration are more convenient for general purposes, the next study evaluated whether the flumazenil-induced enhancement of clonidine antinociception could be seen following systemic administration of the compounds. Clonidine was given IP at a dose that produced little antinociception (Fig. 4A). However, a significant enhancement of the antinociceptive response for IP clonidine was obtained when flumazenil was given IP at 20 and 30, but not 60, min before the tail-flick test. This increase in antinociceptive activity for clonidine in the presence of flumazenil was further demonstrated by a shift in the ED_{50} value for IP clonidine-induced antinociception (Fig. 4B). Clonidine given IP in

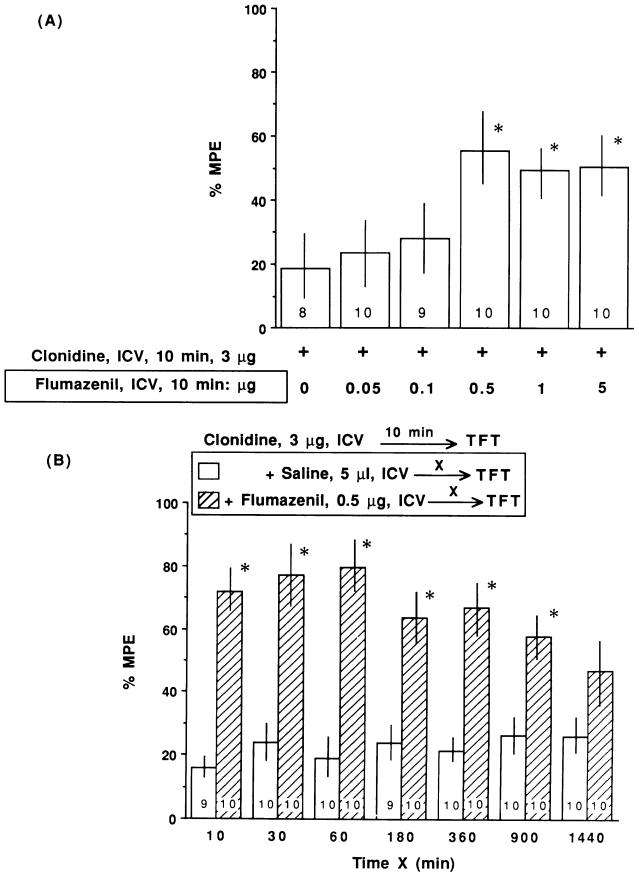

FIG. 3. (A) Administration of flumazenil at doses above 0.5 μg enhanced clonidine-induced antinociception (group 1 vs. 4, 5 and 6). *Indicates that the group is significantly different from the group given clonidine alone (group 1) using Dunnett's test; $p < 0.05$. (B) The antinociceptive activity of clonidine was enhanced by administering flumazenil ICV up to 15 h (900 min) before the tail flick test. *Indicates that the group is significantly different from the time matched control group (group to the immediate left) using Student's *t*-test; $p < 0.05$.

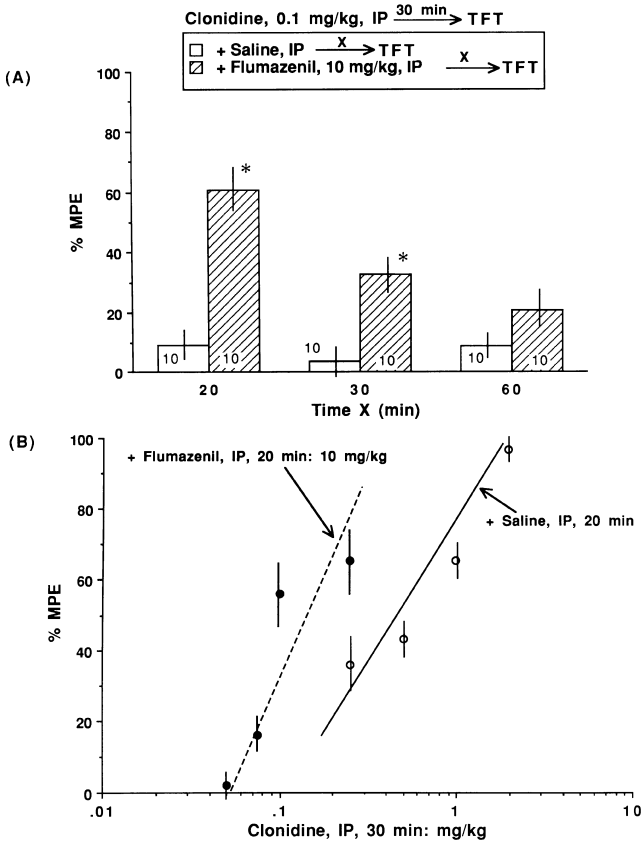

FIG. 4. (A) Administration of flumazenil IP at 20 and 30 but not 60 min before the tail flick test produced an increase in the antinociceptive activity of clonidine. *Indicates that the group is significantly different from the time matched control group using Student's *t*-test; $p < 0.05$. (B) Clonidine administered IP produced a dose-dependent antinociceptive response (open circles). Administration of flumazenil IP produced a parallel, leftward shift in the dose–response curve for IP clonidine-induced antinociception (filled circles).

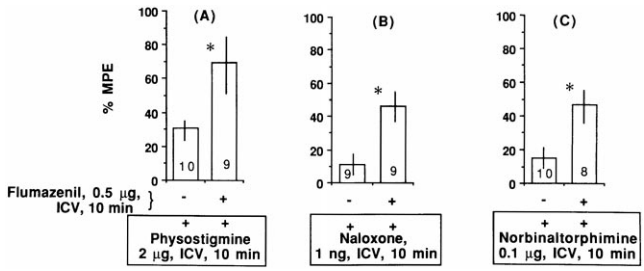

FIG. 5. (A) The antinociceptive response produced by physostigmine given ICV was increased by coadministration of flumazenil (group 1 vs. 2). *Indicates in A, B, and C that the group is significantly different from the control group using Student's *t*-test; $p < 0.05$. (B) Coadministration of flumazenil with ICV naloxone resulted in a significant antinociceptive response (group 1 vs. group 2). (C) Administration of flumazenil along with norbinaltorphimine resulted in antinociception (group 1 vs. 2).

the presence of IP saline produced a dose-dependent antinociceptive response. Administration of flumazenil produced a parallel leftward shift in the clonidine dose–response curve. The ED_{50} value (95% confidence interval) changed from 0.47 (0.29–0.75) mg/kg to 0.15 (0.10–0.23) mg/kg in the presence of flumazenil, representing a threefold leftward shift.

ICV Flumazenil Enhances the Antinociceptive Action of Other Agents (Physostigmine, Naloxone, Norbinaltorphimine) That Release Spinal Dyn When Given ICV

Physostigmine administered ICV not only produces analgesia but also releases Dyn (17). The antinociceptive response produced by ICV physostigmine was increased following administration of flumazenil ICV (Fig. 5A).

The opioid antagonists, naloxone and norbinaltorphimine, given ICV produce an antianalgesic action through release of spinal Dyn (21). Naloxone administered ICV produced no antinociception (Fig. 5B). When flumazenil was administered with naloxone a significant antinociceptive response occurred. Similarly, ICV administration of flumazenil with the ICV norbinaltorphimine produced a significant antinociceptive effect compared to ICV norbinaltorphimine by itself (Fig. 5C). Therefore, ICV flumazenil uncovered the antinociceptive action of naloxone and norbinaltorphimine given ICV.

GABA Receptor Activation and Benzodiazepine Receptor Inverse Agonists Are Not Involved in Dyn Antianalgesia

Because flumazenil is a benzodiazepine receptor antagonist and $GABA_A$ receptors are involved in the pharmacological action of benzodiazepines, the next study considered whether antagonists acting on $GABA_A$ receptor function would have the same effect as flumazenil. The results in Fig. 6 show that ICV administration of bicuculline, a $GABA_A$ receptor antagonist (8,42), along with IT Dyn, did not significantly alter the antianalgesic action of Dyn against morphine-induced antinociception. Similarly, the antianalgesic action of

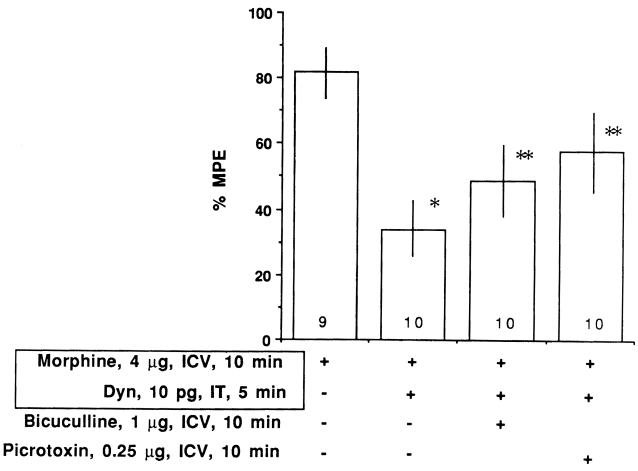

FIG. 6. Administration of bicuculline ICV did not affect the antianalgesic action of IT Dyn against ICV morphine (group 2 vs. 3). This action of Dyn was also not affected by ICV coadministration of picrotoxin with the morphine (group 2 vs. 4). *Indicates that the group is significantly different from the morphine only group, $p < 0.05$, and *indicates that the groups are not significantly different from any of the other groups, $p > 0.05$, using ANOVA followed by Newman–Keuls' test.

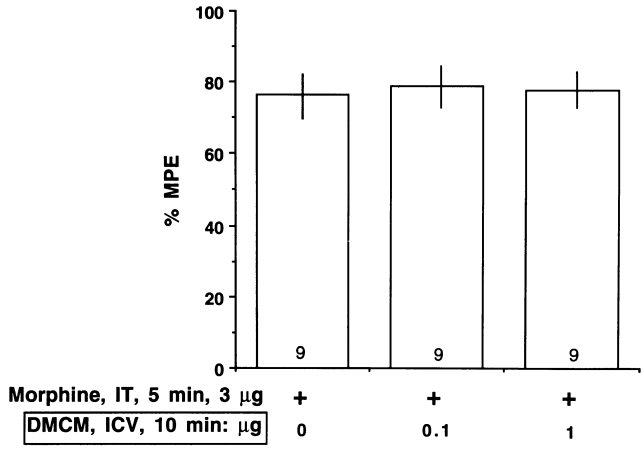

FIG. 7. Administration of DMCM did not alter the antinociceptive response to IT morphine, $F(2, 24) = 0.05$, $p > 0.05$.

IT Dyn against morphine antinociception was not affected significantly by ICV administration of picrotoxin, a noncompetitive chloride ion channel inhibitor (42). These doses of bicuculline and picrotoxin were shown previously to shorten the duration of loss of the righting reflex produced by pentobarbital given IP (50). It should be noted, however, that the bicuculline- and picrotoxin-treated groups were not significantly different from the group that received only morphine, and gave a response that was between that of the morphine and morphine plus Dyn groups.

The possibility that flumazenil was antagonizing the action of a benzodiazepine receptor inverse agonist was considered next. The results in Fig. 7 indicated that DMCM, an inverse agonist, did not have an antianalgesic action against IT morphine. Thus, flumazenil was not antagonizing an action of an endogenous inverse agonist. The results in Fig. 8 indicated that DMCM given ICV inhibited the antianalgesic action of

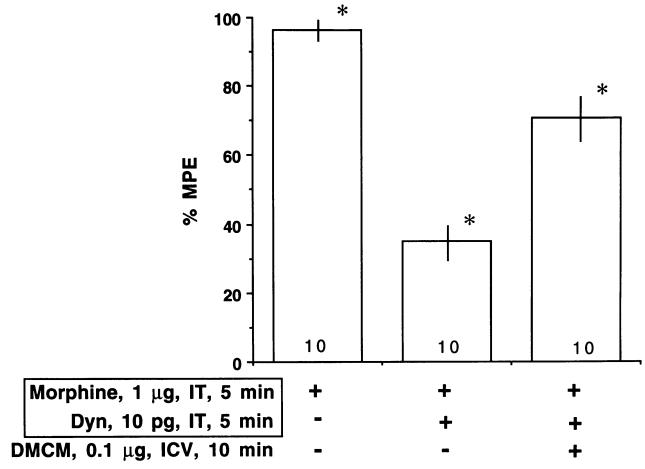

FIG. 8. Administration of DMCM inhibited the antagonistic effect of IT Dyn against IT morphine (group 2 vs. 3). *Indicates that the group is significantly different from the other two groups using ANOVA followed by Newman–Keuls' test; $p < 0.05$.

Dyn. It is possible that flumazenil acted as an inverse agonist to antagonize Dyn antianalgesia.

DISCUSSION

Dyn given IT decreases the antinociception induced by a number of agents given ICV (14–17) and morphine given IT (13,38). The present study first examined the ability of IT Dyn to shift the dose–response curves for ICV and IT morphine-induced antinociception. For morphine by itself, a peculiarity was noted in that the dose–response curves had an inverted U-shape. In our experience with ICR mice, we have not seen this shape of curve because administration of higher doses of morphine are usually not given ICV and IT (40,46,47). In one previous case, we (2) observed an inverted U-shaped curve for ICV DAMGO-induced antinociception that involved a spinal action of serotonin. Such dose–response curves might also arise from opposite actions of morphine. Depending upon the intracerebral site of administration and the dose of morphine, analgesic and hyperalgesic actions have been found in the rat (25,26). Biphasic actions also occur in the spinal cord and spinal sensory neurons (7).

The dose–response studies for morphine clearly indicated that IT Dyn inhibited the antinociceptive activity of ICV and IT morphine. For ICV morphine, an indirect effect for the IT Dyn is suspected because the drugs are given at separate sites that are remote from each other. Even when morphine and Dyn are both given IT, an indirect mode of Dyn action is suggested. For instance, the dose–response curves for morphine in the presence of Dyn suggested a noncompetitive interaction because the Dyn effect was not overcome by giving higher doses of morphine. The action of morphine given IT to rats in which the sciatic nerve is ligated at L5/L6 produces a maximal response that reaches a plateau at about 60% MPE (35). This reduced efficacy of IT morphine is postulated to involve Dyn. Furthermore, as mentioned previously, spinal transection in mice eliminates the Dyn antianalgesia without altering IT morphine antinociception (51). Thus, the Dyn activates a system that involves supraspinal modulation to produce the antianalgesic effect in the present situation. The aim now was to show that flumazenil given ICV inhibits the antianalgesic action of spinal Dyn.

In single-dose studies with ICV morphine-, DPDPE-, and IT morphine-induced antinociception Dyn given IT inhibited the antinociceptive actions. This effect of Dyn was reversed by administration of flumazenil ICV. In a more thorough dose–response study of ICV and IT morphine, the nonparallel shifts to the right produced by IT Dyn were reversed by ICV flumazenil. For the most part, the ICV flumazenil brought the morphine curves back to their original position. However as noted, at the 0.1 μg dose of morphine given IT, the ICV flumazenil plus Dyn group % MPE was significantly greater than that for IT morphine alone. A possible explanation for this is that a small antianalgesic component exists in the action of ICV and IT morphine (16,21), which has been demonstrated at only the lower doses of morphine. Inhibition of the antianalgesic action of this endogenously released Dyn is seen as an enhancement of morphine analgesia, but when morphine analgesia is maximal, further enhancement cannot be seen. Regardless of this, flumazenil inhibited the antianalgesic action of Dyn administered IT. The possibility that flumazenil administered ICV might reach the spinal cord to inhibit Dyn antianalgesia was also considered. If flumazenil were to act on the cord, IT administration of flumazenil should produce effects similar to that of ICV administration. The present results indicated that flumazenil administered IT lacked the activity observed with ICV flumazenil, suggesting that flumazenil acts in the brain and not in the spinal cord to inhibit Dyn antianalgesia.

Flumazenil has been reported to have antinociceptive activity (33). In rats this appears to be biphasic in that low doses of flumazenil given IP produces analgesia while there is a delay for the appearance of analgesia following administration of high doses of flumazenil IP. Therefore, it is possible that the above effect may be due to antinociception produced by flumazenil. However, in the present study, flumazenil administered ICV (at a dose that inhibited the Dyn effect and increased clonidine antinociception) did not induce an antinociceptive response by itself, a finding supported by work of Zambotti et al. (53)

Because flumazenil inhibits the antianalgesic action of IT Dyn, it would be expected that the antianalgesic action of endogenously released Dyn would be inhibited as well. Clonidine administered ICV produces limited antinociception due to the presence of the Dyn antianalgesic action (13–15,38). Administration of increasing doses of flumazenil ICV produced a significant increase in the antinociceptive response obtained with ICV clonidine. This enhancing effect of flumazenil was not dose dependent. At a dose of 0.5 μg and above, an increase in effect was not found. This plateau effect would be understandable in that the dose of clonidine ICV was fixed and thus limited the amount of Dyn released. The ability of flumazenil to increase the antinociceptive activity of clonidine lasted up to 15 h. Most reports suggest that flumazenil is shorter acting, at least on systemic administration, due to rapid metabolic inactivation (4,31,32). It is not known why the duration of action of ICV flumazenil was long when following IP administration, it was less than 1 h. Nevertheless, these results demonstrated that ICV flumazenil inhibited the antianalgesic action of Dyn released endogenously in the spinal cord.

Physostigmine, which has antinociceptive properties, also releases spinal Dyn (14,15,17). The finding that the antinociceptive response to ICV physostigmine was enhanced by coadministration of flumazenil was again consistent with flumazenil inhibiting the antianalgesic action of the endogenously released spinal Dyn. The final study was based on the previous finding that naloxone and norbinaltorphimine given ICV produce an antianalgesic action through release of spinal Dyn (21). If flumazenil given ICV inhibits Dyn action, the antinociceptive action of naloxone and norbinaltorphimine should become evident. The results were consistent with this idea. Flumazenil given ICV uncovered the antinociceptive action of naloxone and norbinaltorphimine. The finding that flumazenil uncovers the antinociceptive action of naloxone is consistent with the observations of Cappell et al. (5). Norbinaltorphimine has also been reported to have slight antinociceptive activity, which is not naloxone reversible, in the writhing test (49). Flumazenil administration results in increased analgesic activity for agents that release Dyn in the spinal cord. Enhancement of anxiety-induced analgesia is also reported to occur following flumazenil treatment (29).

It should be emphasized that the action of flumazenil was in the brain, while the action of Dyn whether released spinally or administered IT was initiated in the spinal cord. Dyn administered ICV does not have an antianalgesic action (unpublished data). The situation in this model is consistent with the finding that the antianalgesic action of Dyn is mediated through an ascending pathway to the brain. The fact that, in the present study, the site of action of flumazenil was in the brain has further implications. Because flumazenil is a benzo-

diazepine receptor antagonist (4,27,31), it appears that the antianalgesic action of spinal Dyn occurs through the activation of benzodiazepine receptors in the brain (41). The conjecture would be that spinal Dyn activates a pathway to the brain that involves release of an endogenous benzodiazepine within the brain. Benzodiazepine receptors are localized in the brain (32,43), and limited evidence suggests that benzodiazepine-like substances are present as well (3,6,34). Also, a benzodiazepine receptor ligand released in the brain by activation of the Dyn antianalgesic system may inhibit the antinociceptive pathways, thus producing the antagonistic effects. Studies suggest that benzodiazepines decrease norepinephrine and serotonin release from central neurons to produce anxiolytic and sedative effects [see review (44)]. Decrease of norepinephrine and serotonin release could lead to antianalgesic effects because descending noradrenergic and serotonergic systems are the major antinociceptive pathways involved in suppressing the tail-flick response (2,11,52).

Due to the allosteric association of the benzodiazepine receptor with the $GABA_A$ receptor (which forms a chloride ion channel), interaction of benzodiazepine agonists with the benzodiazepine receptor alters GABA activity, which ultimately changes chloride ion flux [see review (36)]. Therefore, inhibition of GABA receptor function might alter the Dyn antianalgesic system. In the present study, however, ICV administration of a competitive $GABA_A$ receptor antagonist (bicuculline) and a chloride ion channel blocker (picrotoxin) did not significantly reverse but had an intermediate effect on the antianalgesic action of IT Dyn. Thus, the possibility of involvement of GABA receptors cannot be eliminated. However, the same doses of the antagonists (bicuculline and picrotoxin) that were used in this study have previously been shown to reverse a GABAergic function of pentobarbital (loss of righting reflex) but did not affect the antianalgesic action of ICV pentobarbital (50). The finding that antianalgesic action of IT Dyn and ICV pentobarbital is inhibited by ICV flumazenil but perhaps not picrotoxin and bicuculline might indicate that an endogenous inverse agonist (6,36,39) rather than a benzodiazepine agonist is involved. An inverse agonist would act by decreasing GABA activity producing an action opposite to that of the benzodiazepine agonists (36). Then, flumazenil would antagonize the inverse agonist action but blockade of GABA receptor action by bicuculline and picrotoxin would not be expected to alter the effect of the ligand released by Dyn. However, in this study, as has been previously reported by others (24), administration of a benzodiazepine receptor inverse agonist, DMCM, had no effect on morphine-induced antinociception (no antianalgesic action). The latter result suggests that the putative endogenous benzodiazepine ligand released by Dyn is not a benzodiazepine inverse agonist.

Flumazenil has also been reported to produce inverse agonist actions itself (12,36). Administration of DMCM ICV inhibited the antagonistic action of IT Dyn against IT morphine, suggesting that flumazenil may be behaving as an inverse agonist. However, the present studies are not sufficient for choosing any one mechanism by which flumazenil acts to attenuate the antianalgesic actions of spinal Dyn.

Finally, a practical implication arises from the interaction found between systemic administration of clonidine and flumazenil. Clonidine is used clinically to treat hypertension and opiate withdrawal (18,19,28). Thus, it seems worthwhile to determine whether flumazenil will selectively increase the antinociceptive activity of clonidine by eliminating the Dyn component without an enhancement of the antihypertensive or other side effects.

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