The beta-carboline DMCM produces hypoalgesia after central administration

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DMCM (Methyl 6, 7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate), an "inverse agonist" at benzodiazepine receptors, produced a dose-related (0.5–20 $\mu\text{g}/\text{rat}$) elevation in the latency of rats to respond to a noxious thermal stimulus 20 min after i.c.v. administration. By 50 min after administration, no hypoalgesia could be detected. In animals given 5.0 μg of DMCM centrally, systemic injection of the opioid antagonist naltrexone HCl (0.75–7.0 mg/ml/kg) resulted in significant attenuation of hypoalgesia only at the highest dose tested. These results are discussed in terms of the role played by GABAergic central mechanisms in the activation of endogenous pain control systems.

A number of synthetic compounds modulate GABAergic activity through the benzodiazepine (BZP) recognition site on the GABA_A receptor complex (Martin, 1987). Agonists, such as diazepam, enhance the inhibitory postsynaptic effect of GABA (Chang & Farb, 1985) and are behaviorally characterized as anxiolytic, both clinically and in animal models of anxiety (Dantzer, 1977; Shepard, 1986). Inverse agonists at the BZP receptor, on the other hand, tend to reduce the efficacy of GABAergic transmission (Lehoullier & Ticku, 1987). Inverse agonists produce behavioral effects that often are consistent with their pharmacological action's being opposite to that of BZP receptor agonists. While agonists at BZP receptors have long been described as anticonvulsant, hyperphagic, and anxiolytic, the inverse agonists tend to be convulsant, hypophagic, and anxiogenic (Cooper & Estall, 1985; Croucher, DeSarro, Jensen, & Meldrum, 1984; Dorrow & Duka, 1986; File, Lister, & Nutt, 1982; Petersen, 1983).

Evidence suggesting that BZP-receptor inverse agonists contribute to the generation of an anxiety-like central process comes from a number of areas. Peripheral administration of inverse agonists can mimic the effects of inescapable shock in producing response deficits similar to those seen in "learned helplessness" (Drugan, Maier, Skolnick, Paul, & Crawley, 1985), and produce dramatic increases in local cerebral glucose utilization in limbic structures often ascribed a role in "emotional" behavior (Ableitner & Herz, 1987). Both inverse agonists and foot-

shock stress cause a similar decrease in low affinity GABA binding in rat brain (Corda & Biggio, 1986). Inverse agonists will enhance the punishing effects of footshock (Duka & Stephens, 1986), selectively increase the number of ultrasonic distress vocalizations made by rat pups (Gardner & Budhram, 1987), and decrease social interaction between conspecifics (File et al., 1982).

When confronted with certain stressful or anxiety-provoking events, animals will often respond with, among other things, an increased threshold for behavioral responses to noxious stimuli (Fanselow, 1986; Hayes, Bennett, Newlon, & Mayer, 1978; Maier, 1986). This hypoalgesia may be observed not only in the presence of an unconditional stressor such as footshock, but also after presentation of once neutral stimuli that have been paired with aversive events during Pavlovian conditioning (Chance, 1980; Fanselow, 1986). The literature on conditional hypoalgesia would suggest that, in most cases, conditional stimuli capable of producing overt fear-related responses such as suppression of ongoing operant responding or defensive freezing are also capable of producing hypoalgesia (Fanselow, 1986). Due to the strong covariation observed between hypoalgesia and other behaviors, it has been proposed that a single central process, linked to an organism's expectancy of noxious stimulation and sometimes described as an emotional state, anxiety, or fear, is responsible for both antinociception and other behavior in aversive situations (Chance, 1980; Fanselow, 1986; Fanselow & Helmstetter, 1988).

Manipulation of GABAergic neurons via the BZP recognition site can also modulate reactions to painful stimuli. BZP-receptor agonists such as diazepam and midazolam completely or partially reverse hypoalgesia produced by exposure to tail shock (Doi & Sawa, 1980), "social defeat" (Rodgers & Randall, 1987), and lesion-induced hyperemotionality (Chance, Krynock, & Rosecrans, 1978). Both intracranial and systemic administration of BZP-receptor agonists attenuate hypoalgesia after exposure to stimuli that predict footshock (Fanselow & Helmstetter,

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1988; Helmstetter, Leaton, Calcagnetti, & Fanselow, 1988).

If the GABA/BZP complex is involved in the modulation of anxiety or fear, and compounds that facilitate GABAergic transmission via the BZP site attenuate hypoalgesia in response to stress, then inverse agonists that seem to promote stress or anxiety in animals might also produce hypoalgesia. Consistent with this idea, Rodgers and Randall (1987) reported that BZP-receptor inverse agonists elicit hypoalgesia in mice when tail-flick latencies are used as an index of pain sensitivity. We conducted two experiments to address this question, using rats in the hotplate test.

EXPERIMENT 1

Experiment 1 was designed to determine the dose-effect relationship for centrally administered DMCM, a potent inverse agonist with a high degree of selectivity for BZP receptors (Braestrup, Schmiechen, Neef, Nielsen, & Petersen, 1982; Petersen, 1983).

Method

Subjects. A total of 45 female Long-Evans rats born and raised in the Dartmouth Psychology Department colony served as subjects. All rats were individually housed and maintained on ad-lib lab chow and water and a 14:10-h light:dark cycle throughout the experiment. Test procedures were conducted during the light portion of the cycle. The rats weighed 210–270 g just prior to surgery and were naive to all aspects of the present test procedure. At least 1 week prior to testing, the rats used in this experiment had received a single i.c.v. injection of the opioid antagonist MR2266 BS ((-)-(1R,5R,9R)-5,9-Diethyl-2-(3-furylmethyl)-2'-hydroxy-6,7-benzomorphan) or its vehicle in a prior study in which no nociceptive testing was done. Assignment to groups in the present study was completely counterbalanced with respect to this prior treatment.

Surgery. Animals were anesthetized with ketamine HCl (100 mg/ml/kg i.p.) followed by sodium pentobarbital (2.5 mg/rat i.p.). Each rat was then implanted with a 22-gauge stainless steel guide cannula aimed at the right lateral ventricle (AP -0.5, L +1.5, V -3.2, relative to bregma with the skull level). The rats were allowed to recover from surgery for at least 1 week, during which they were adapted to transportation and handling. During this period, they had their cannula obturators removed, cleaned with 70% methanol, and replaced each day.

At the conclusion of the experiment, each animal was overdosed with sodium pentobarbital, injected i.c.v. with 2 μ l india ink solution, and 15 min later perfused transcardially with saline followed by buffered 10% formalin. Coronal sections were made along the cannula track, and cannula placement was verified by the presence of ink throughout the cerebral ventricles. Only animals showing positive cannula placement were included in the analysis.

Drugs. DMCM (Methyl 6, 7-dimethoxy-4-ethyl- β -carboline-3-carboxylate, a gift of D. N. Stephens, Schering AG) was first dissolved in 0.1 N HCl and eventually brought to a final pH of 3.80 with NaOH and pyrogen filtered distilled water. The drug was prepared in concentrations of 0.5, 1.0, 2.5, 10.0, and 20.0 μ g/4 μ l. Pyrogen filtered isotonic saline adjusted to pH 3.80 served as a control. Injections were performed at a rate of 4 μ l/15 sec via a 28-gauge injection cannula connected to a 10- μ l Hamilton syringe with PE 50 tubing.

Apparatus and Procedure. Testing took place on a 26.5 $\times$ 27.5 cm copper hotplate (IITC, Inc., Landing, NJ, Model 35-D) set at 52°C. The surface of the hotplate was surrounded by Plexiglas walls 45 cm high on each side. Approximately 20 min after the i.c.v. injection, each animal was placed on the hotplate, and its latency to raise a rear paw from the hotplate surface and place it in contact with its mouth was recorded. In the event that an animal jumped from the surface of the hotplate, where a jump was defined as having all four paws off the surface simultaneously, it was assigned a score equal to its latency to jump. If neither of these behaviors were observed within 90 sec, a score of 90 sec was recorded. Each animal was then tested in this manner a second time, 50 min after drug administration. The experimenter was blind to group assignments throughout testing.

Results

Since the paw-lick scores were characterized by a gamma distribution, the raw data were transformed by taking the natural logarithm of each subject's latency in order to approximate a normal distribution prior to analysis of variance (ANOVA). Mean log paw-lick latencies for groups of animals given central injections of DMCM or vehicle 20 min prior to testing may be seen in Figure 1. As can be seen in the figure, animals given larger doses of DMCM tended to display elevated lick latencies on the hotplate relative to controls. This observation was supported by a one-way ANOVA performed on transformed scores that indicated a reliable main effect for DMCM dose [$F(5,39) = 3.48, p < .02$]. In addition, a subsequent trend test confirmed a linear dose-effect relationship [$F(1,39) = 15.05, p < .001$]. The asterisks in Figure 1 denote groups differing significantly from controls, on the basis of individual planned comparisons made with $\alpha = 0.01$.

At 50 min after injection of DMCM, paw-lick latencies did not differ reliably [$F(5,39) < 1$]. Transformed means for drug-treated groups ranged from 2.33 to 2.41, while the mean for controls was 2.58. Thus, no drug-related effect could be detected 50 min after administration.

EXPERIMENT 2

Considerable evidence suggests that conditional hypoalgesia, in which an animal's pain sensitivity is altered in the presence of "fear-provoking" cues that signal a noxious unconditioned stimulus, is dependent on CNS opioid systems (Calcagnetti, Helmstetter, & Fanselow, 1987; Helmstetter & Fanselow, 1987; Watkins & Mayer, 1982). Therefore, if DMCM is able to produce hypoalgesia through an anxiogenic influence on a central system involved in emotional responses, and this same system plays a role in normal responses to conditional aversive stimuli (i.e., conditional hypoalgesia), then the hypoalgesia produced by DMCM should also be dependent on endogenous opioid systems.

To address this question we administered the opioid antagonist naltrexone HCl systemically after a central in-

Figure 1. Hypoalgesic dose-effect curve for centrally administered DMCM. Doses are expressed in micrograms per rat. Scores represent the natural logarithm of the latency for an animal to lick its hind paw after being placed on a copper hotplate maintained at 52°C. Asterisks indicate a reliable difference from controls ($p < .01$). $N = 7$ or 8 animals per group.

jection of DMCM to determine if endogenous opioid antinociceptive systems were involved.

Method

Subjects. A total of 20 naive female Long-Evans rats similar to those described in Experiment 1 served as subjects. The rats were prepared with ventricular cannulae and adapted to transportation and handling as previously described. The rats were naive to all aspects of the procedure prior to testing.

Procedure. The rats were randomly assigned to 4 groups scheduled to receive 0.75, 2.3, or 7.0 mg/ml/kg naltrexone HCl, or the saline vehicle. All animals were given an i.c.v. injection containing 5 µg of DMCM dissolved in 4 µl dimethyl sulfoxide (DMSO) followed immediately by an i.p. injection of naltrexone or saline. DMSO was used as a vehicle in the present experiment to improve the uniformity of in vivo distribution of the drug and to eliminate the need for the acidic vehicle. Ten minutes after drug treatment, all the rats were tested on the hotplate as described above.

Results

Figure 2 depicts the results of combined administration of DMCM and naltrexone. One animal was rejected prior to analysis, because of incorrect cannula placement. An ANOVA on transformed latencies indicated a reliable main effect for naltrexone treatment [$F(3,15) = 3.90$, $p < .03$]. Subsequent planned comparisons indicated that the 7.0-mg/kg doses of naltrexone differed reliably from saline ($p < .01$), whereas the two intermediate doses did not. There was no linear relationship between hotplate latency and naltrexone dose.

DISCUSSION

The present study demonstrates that central administration of DMCM, an inverse agonist at the BZP recep-

tor, can dose-dependently elevate hotplate response latencies in rats. These results are consistent with the existence of a BZP-receptor-sensitive process involved in analgesic responses to aversive motivational states. Thus, BZP-receptor inverse agonists may provoke defensive behavior and produce changes in nociceptive reactivity through disinhibition of forebrain structures currently thought to be involved in the generation of fear or anxiety, as well as the consequent activation of descending brainstem systems that modulate sensory input at the level of the spi-

Figure 2. The data from Experiment 2, expressed as the mean natural logarithm of paw-lick latencies for each group and recorded 10 min after drug treatment. DMCM (5 µg) was centrally administered. Naltrexone was injected i.p. immediately thereafter. $N = 4$ or 5 animals per group.

nal cord. Recent work in our laboratory indicates that BZP receptors in the limbic forebrain and opioid receptors in the periaqueductal gray (PAG) may be critically involved (Helmstetter, 1989; Helmstetter & Landieria-Fernandez, 1989).

Another account of the present findings should also be considered. DMCM may act directly on certain brainstem nuclei and activate descending antinociceptive systems independently of any anxiogenic effect. GABAergic manipulation of cells within the PAG, nucleus raphe magnus and nucleus reticularis gigantocellularis pars alpha, has been shown to affect nociceptive reactivity (see, e.g., Drower & Hammond, 1988; Moreau & Fields, 1986). According to the model proposed by Moreau and Fields (1986), GABAergic interneurons in the PAG tonically inhibit "output cells" that ultimately influence responding. Local opioid neurons inhibit the GABAergic cells, in turn releasing the output cells from inhibition. The present results, when viewed from this perspective, would indicate that DMCM acting as a GABA antagonist within the PAG produces hypoalgesia through a release of inhibition on PAG cells projecting into the rostral medulla. Since in the simplest case this model predicts a lack of naltrexone reversibility because opioid effects should be functionally presynaptic to GABAergic modulation, one could argue that the antagonism of hypoalgesia observed at our highest naltrexone dose reflects an action on opioid receptors in another part of the system. Receptors within the spinal cord would be one possibility.

It is also possible that DMCM could be elevating hotplate latencies but not actually changing nociceptive thresholds. The drug may simply have interfered with the animals' ability to perform the paw-lick response. However, our informal behavioral observations fail to support an account solely in terms of secondary drug effects. While DMCM is a potent convulsant (Petersen, 1983), only 1 animal in Experiment 1 exhibited brief clonic jerks within 10 min of receiving the 20- μ g dose. The remaining animals evidenced no obvious convulsions or motor impairment, although the drug-treated rats did vocalize more frequently when handled. Furthermore, partial reversibility of this elevation in hotplate latency by naltrexone supports the idea that DMCM is operating via the interaction of multiple neurochemical systems rather than directly disrupting the animals' ability to perform the response.

Naltrexone antagonism of DMCM-induced antinociception was observed only at the highest dose (7 mg/kg) tested. Although this dose of naltrexone is sufficient to produce a comparable attenuation of conditional hypoalgesia (see, e.g., Helmstetter & Fanselow, 1987), one would expect a graded dose-response function over the concentrations used here. It may be that our failure to show a naltrexone effect at lower doses is due to a "ceiling effect" in Experiment 2. The 5- μ g concentration of DMCM was more effective in elevating latencies in Experiment 2, most likely because (1) DMCM dissolved in DMSO had better access to critical brain structures surrounding the

cerebral ventricles, and (2) animals were tested at 10 min instead of 20 min after drug administration. Thus, the 90-sec cutoff employed during hotplate testing may have partially masked the dose-effect function for naltrexone. It is important to note that doses of naltrexone equal to or greater than 7 mg/kg are required to block certain forms of stress-induced hypoalgesia (Maier, 1986). Further work will be required to determine in detail the nature of opioid/benzodiazepine interactions in this preparation.

In conclusion, DMCM produced hypoalgesia after central administration. This hypoalgesia was reversed in animals given a dose of naltrexone that had previously been shown to be effective in attenuating hypoalgesia in response to Pavlovian signals for footshock. Further research will be needed to determine both the pharmacological selectivity of DMCM's effect with respect to the GABA/BZP receptor complex and the relative importance of forebrain versus brainstem loci of action.

REFERENCES

- ABLEITNER, A., & HERZ, A. (1987). Changes in local cerebral glucose utilization induced by the B-carbolines FG 7142 and DMCM reveal brain structures involved in the control of anxiety and seizure activity. *Journal of Neuroscience*, **7**, 1047-1055.
- BRAESTRUP, C., SCHMIECHEN, R., NEEF, G., NIELSEN, M., & PETERSEN, E. N. (1982). Interaction of convulsive ligands with benzodiazepine receptors. *Science*, **216**, 1241-1243.
- CALCAGNETTI, D. J., HELMSTETTER, F. J., & FANSELOW, M. S. (1987). Quaternary naltrexone reveals the central mediation of conditional analgesia. *Pharmacology, Biochemistry & Behavior*, **27**, 529-531.
- CHANCE, W. T. (1980). Autoanalgesia: Opiate and nonopiate mechanisms. *Neuroscience & Biobehavioral Reviews*, **4**, 55-67.
- CHANCE, W. T., KRYNOCK, G. M., & ROSECRANS, J. A. (1978). Antinociception following lesion-induced hyperemotionality and conditioned fear. *Pain*, **4**, 243-252.
- CHANG, C. Y., & FARB, D. H. (1985). Modulation of neurotransmitter action: Control of the gamma-aminobutyric acid response through the benzodiazepine receptor. *Journal of Neuroscience*, **5**, 2365-2373.
- COOPER, S. J., & ESTALL, L. B. (1985). Behavioral pharmacology of food, water, and salt intake in relation to drug actions at benzodiazepine receptors. *Neuroscience & Biobehavioral Reviews*, **9**, 5-19.
- CORDA, M. G., & BIGGIO, G. (1986). Stress and GABAergic transmission: Biochemical and behavioral studies. In G. Biggio & E. Costa (Eds.), *GABAergic transmission and anxiety* (pp. 121-136). New York: Raven Press.
- CROUCHER, M., DESARRO, G., JENSEN, L., & MELDRUM, B. (1984). Behavioral and convulsant actions of two methyl esters of Beta-carboline -3- carboxylic acid in photosensitive baboons and in DBA/2 mice. *European Journal of Pharmacology*, **104**, 55-60.
- DANTZER, R. (1977). Behavioral effects of benzodiazepines: A review. *Biobehavioral Reviews*, **1**, 71-86.
- DOI, T., & SAWA, N. (1980). Antagonistic effects of psycholeptic drugs on stress-induced analgesia. *Archives Internationales de Pharmacodynamie et de Therapie*, **247**, 264-274.
- DORROW, R., & DUKA, T. (1986). Anxiety: Its generation by drugs and their withdrawal. In G. Biggio & E. Costa (Eds.), *GABAergic transmission and anxiety* (pp. 211-225). New York: Raven Press.
- DROWER, E. J., & HAMMOND, D. L. (1988). GABAergic modulation of nociceptive threshold: Effects of THIP and bicuculline microinjected in the ventral medulla of the rat. *Brain Research*, **450**, 316-324.
- DRUGAN, R. C., MAIER, S. F., SKOLNICK, P., PAUL, S. M., & CRAWLEY, J. N. (1985). An anxiogenic benzodiazepine receptor ligand induces learned helplessness. *European Journal of Pharmacology*, **113**, 453-457.

- DUKA, T., & STEPHENS, D. N. (1986). Potentiation of the propunishment, but not the convulsant action of the B-carboline DMCM by naltrexone. *Pharmacology, Biochemistry & Behavior*, **25**, 595-598.
- FANSELOW, M. S. (1986). Conditioned fear-induced opiate analgesia: A competing motivational state theory of stress-induced analgesia. *Annals of the New York Academy of Sciences*, **467**, 40-54.
- FANSELOW, M. S., & HELMSTETTER, F. J. (1988). Conditional analgesia, defensive freezing and benzodiazepines. *Behavioral Neuroscience*, **102**, 233-243.
- FILE, S. E., LISTER, R. G., & NUTT, D. J. (1982). The anxiogenic action of benzodiazepine antagonists. *Neuropharmacology*, **21**, 1033-1037.
- GARDNER, C. R., & BUDHRAM, P. (1987). Effects of agents which interact with central benzodiazepine binding sites on stress-induced ultrasonics in rat pups. *European Journal of Pharmacology*, **134**, 275-283.
- HAYES, R. L., BENNETT, G. J., NEWLON, P. G., & MAYER, D. J. (1978). Behavioral and physiological studies of non-narcotic analgesia in the rat elicited by certain environmental stimuli. *Brain Research*, **155**, 69-90.
- HELMSTETTER, F. J. (1989). *Forebrain mechanisms of conditional hypoalgesia: Involvement of the amygdala*. Unpublished PhD dissertation, Dartmouth College, Hanover, NH.
- HELMSTETTER, F. J., & FANSELOW, M. S. (1987). Effects of naltrexone on learning and performance of conditional fear-induced freezing and opioid analgesia. *Physiology & Behavior*, **39**, 501-505.
- HELMSTETTER, F. J., & LANDERIA-FERNANDEZ, J. (1989). Conditional hypoalgesia on the formalin test is blocked by naltrexone applied to the PAG. *Society for Neuroscience Abstracts*, **15**, 155.
- HELMSTETTER, F. J., LEATON, R. N., CALCAGNETTI, D. J., & FANSELOW, M. S. (1988). The amygdala is involved in the expression of conditional analgesia. *Society for Neuroscience Abstracts*, **14**, 1227.
- LEHOULLIER, P. F., & TICKU, M. K. (1987). Benzodiazepine and B-carboline modulation of GABA-stimulated ^{36}Cl -influx in cultured spinal cord neurons. *European Journal of Pharmacology*, **135**, 235-238.
- MAIER, S. F. (1986). Stressor controllability and stress-induced analgesia. *Annals of the New York Academy of Sciences*, **467**, 55-72.
- MARTIN, I. L. (1987). The benzodiazepines and their receptors: 25 years of progress. *Neuropharmacology*, **26**, 957-970.
- MOREAU, J., & FIELDS, H. L. (1986). Evidence for GABA involvement in midbrain control of medullary neurons that modulate nociceptive transmission. *Brain Research*, **397**, 37-46.
- PETERSEN, E. N. (1983). DMCM: A potent convulsive benzodiazepine receptor ligand. *European Journal of Pharmacology*, **94**, 117-124.
- RODGERS, R. J., & RANDALL, J. I. (1987). Benzodiazepine ligands, nociception and 'defeat' analgesia in male mice. *Psychopharmacology*, **91**, 305-315.
- SHEPARD, R. A. (1986). Neurotransmitters, anxiety and benzodiazepines: A behavioral review. *Neuroscience and Biobehavioral Reviews*, **10**, 449-461.
- WATKINS, L. R., & MAYER, D. J. (1982). Organization of endogenous opiate and nonopiate pain control systems. *Science*, **216**, 1185-1192.

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