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Effects of Morphine and Two Enzyme Resistant Enkephalins on Schedule-Controlled Responding in the Rat[1]

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CARNEY, J. M. AND J. A. ROSECRANS. *Effects of morphine and two enzyme resistant enkephalins on schedule-controlled responding in the rat.* PHARMAC. BIOCHEM. BEHAV. 8 (2) 185–189, 1978. – Water-deprived rats to respond for access to a water-filled dipper under a 20 response fixed-ratio schedule. After training, the rats were each implanted with a lateral ventricle cannula and the effects of intraventricular injection of morphine, (N-methyl-tyrosyl)[1]-des COOH-Norleucyl[5]-Enkephalin, and (D-Ala)[2] - (des COOH-Norleucyl)[5]-Enkephalin were determined. All three compounds produced dose-related decreases in FR20 responding. Morphine was about 5 times more potent than either of the two peptides. Naloxone (1.0 mg/kg, SC) antagonized the response-rate decreasing effects of intraventricular morphine and the two opiate peptides.

Opiate peptides Morphine Enkephalins Intraventricular injections
Schedule-controlled responding Water reinforcement Naloxone antagonism

A WIDE variety of opiates have been reported to produce dose-related decreases in schedule-controlled responding in pigeons, rats and monkeys [4, 9, 10, 12, 17, 23, 31, 32]. These narcotic-induced changes in responding have been demonstrated to be 'specific narcotic effects' as defined by Domino and Wilson [7]. The decreases in responding were antagonized by naloxone and other narcotic antagonists [9, 10, 12, 20, 21, 22]. Chronic administration of morphine or methadone resulted in the development of tolerance to the rate-decreasing effects of the narcotic [4,14]. In addition, morphine-tolerant or methadone-tolerant subjects were cross-tolerant to other opiates [4,14]; but they were not cross-tolerant to non-narcotic isomers of opiates [4]. Thus, schedule-controlled responding is a useful system in which to evaluate the *in vivo* effects of narcotic agonists, antagonists and related compounds.

Recently, Hughes, *et al.* [16] identified two pentapeptides from procine brain which had opiate-like activity in the isolated guinea pig ileum and mouse vas deferens [15,16] assay systems. The pentapeptides, referred to as enkephalins, have also been identified in neural tissue from other animals [28]. In the *in vitro* assay systems for opiates these pentapeptides produce qualitatively similar effects to those produced by morphine. The *in vivo* characterization of Methionine[5]-Enkephalin and Leucine[5]-Enkephalin has been hampered by the relatively short duration of action of these peptides. Belluzzi, *et al.* [1] reported that Met[5]-Enkephalin produced antinociception in rodents, but the duration of this antinociception was only about 2 min. Pert, *et al.* [25] reported the synthesis of an analog of Met[5]-Enkephalin in which the Glycine[2] was replaced by a D-Alanine, which would change the tertiary structure of the peptide and prevent its metabolism by conventional enzymes. This D-Ala[2]-Enkephalin was comparable in potency to Met[5]-Enkephalin in the *in vitro* assays and produced potent, long lasting antinociceptive effects in rats when injected into the peri-aqueductal gray matter of the brain.

The present study was designed to compare the effects of intraventricular injection of morphine, (N-methyl-Tyrosyl)[1]-(des COOH-Norleucyl)[5]-Enkephalin, and (D-Ala)[2]-(des COOH-Norleucyl)-Enkephalin under a 20 response fixed-ratio (FR20) schedule of water reinforcement. The ability of naloxone to antagonize the response-rate decreasing effects of morphine and the two enzyme-resistant enkephalins was also determined.

METHOD

Animals

The animals were 3 male SD rats (Flow-Labs; Dublin,

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MD) which weighed between 250 and 300 g at the start of the study. Rats were individually housed in plastic cages to eliminate the problem of broken cannulae which occurs when rats are housed in the standard metal, wire cages. The animals were housed in a temperature controlled room (25°C) which had a 12/12 light/dark cycle. Rats had free access to food in their home cages throughout the study.

Procedure

Prior to training, rats were deprived of water for a period of 48 hr. After this initial period of water deprivation rats trained to depress a lever for a 5 sec access to a water-filled 0.1 ml dipper under a single response fixed-ratio schedule of responding. Once reliable responding occurred the FR was gradually increased over days to the terminal FR20 schedule. Daily sessions were conducted in a standard operant chamber (Coulbourn Instruments) which was contained within a sound attenuating chamber. Under the terminal schedule conditions the availability of water under the FR20 schedule was signalled by the illumination of a white light over the response lever. Each depression of the response lever resulted in the illumination of a red light for as long as the lever was held down and provided visual feedback. After the 20th response was emitted, the lever lights and the house light were extinguished and the water-filled dipper was presented for a period of 5 sec. A light over the dipper was illuminated at the same time the dipper was raised. Animals were allowed to complete 10 FR20's before injection of either saline or drug. Sessions were terminated 30 min after intraventricular injection of saline or drug. After each daily session the rats were allowed 30 min access to water. Thus, each day rats were tested under a 23 hr water-deprivation condition. Animals were run 7 days a week and sessions were always conducted at the same time each morning. Solid-rate programming and controlling equipment were used in the present study and data was recorded by means of digital response counters and a cumulative event recorder (Ralph Gerbrands, model SHS-3).

Following training each rat was implanted with a lateral ventricle cannula. Rats were pretreated with atropine (5 mg/kg) and anesthetized with a drug combination of chloralhydrate (6%), magnesium sulfate (3%) and pentobarbital (0.65%) (L.A. Thesia®, Haver-Lockhart, Shawnee, KS; 1.0 cc/kg). Each rat was implanted with a modified Kopf cannula (David Kopf Instruments, Tujunga, CA) which has a cannula shaft made of 23 ga hypodermic tubing (Small Parts, Miami, FL). The cannula was placed in the lateral ventricle (A.P. 5.2, LAT. 2.1, D.V. 3.2) and anchored in place with dental acrylic. Injections were made with a 30 ga injection cannula which was connected to a microliter syringe outside of the chamber by means of a polyvinylchloride catheter. The injection catheter was protected from damage by an outer sheath of tubing (Tygon®) which extended from the animals headed to the microliter syringe. The weight of the infusion catheter was minimized by a wire spring suspension system. Intraventricular injection (5 μl) of drug or saline was delivered by means of a repeating dispenser (Hamilton No. PB600-1) and was delivered in 0.5 μl steps. The injections were given at a rate of 1 μl/15 sec. Animals received an intraventricular drug injection every 5th day, which was preceded by a saline injection on the previous day.

Drugs

(D-Ala)^2-(desCOOH-Norleucyl)^5-Enkephalin and (N-methyl-Tyrosyl)^1-(desCOOH-Norleucyl)^5-Enkephalin were synthesized in solution by the classical method [2]. The peptides were purified by counter current distribution using butanol: acetic: water (4:1:5). The purity of each peptide was determined in three different solvent systems using silica gel thin-layer-chromatography. The solvent systems were: (A) Butanol: acetic acid: water (4:1:1); (B) Chloroform: methanol: acetic acid: water (60:30:4:1); and (C) Benzene: acetic acid: water (9:9:1). In each TLC system the peptides chromatographed as a single ninhydrin-positive spot. The purity of the peptide was further demonstrated by amino acid content analysis. The peptides were the generous gift of Drs. R. J. Freer and A. R. Day, Department of Pharmacology, Medical College of Virginia, Richmond, VA 23298. The acetate salts of the peptides were dissolved in a sterile 0.9% saline and the pH adjusted to between 7.0 and 7.4 with NaOH. Naloxone HCl and Morphine SO_4 were also dissolved in sterile saline. Naloxone was the generous gift of Endo Labs. Morphine was purchased commercially. Because of the potential breakdown of the peptides due to bacterial and/or spontaneous hydrolysis, 100 μl aliquots of the peptide solutions were individually frozen in plastic tubes. Vials were separately thawed when needed. All doses are expressed in mg of the base.

RESULTS

The average fixed-ratio rate of water reinforced responding for the three rats was 1.26 resp/sec (Fig. 1). Injection of 5 μl of saline did not produce any consistent change in responding when compared to the non-injection controls. Intraventricular injection of morphine, (D-Ala)^2-Enkephalin and N$^\alpha$-methyl-Enkephalin produced dose-related decreases in FR20 responding. Since (D-Ala)^2-Enkephalin and N$^\alpha$-methyl-Enkephalin both had molecular weights of 526 which were substantially greater than that

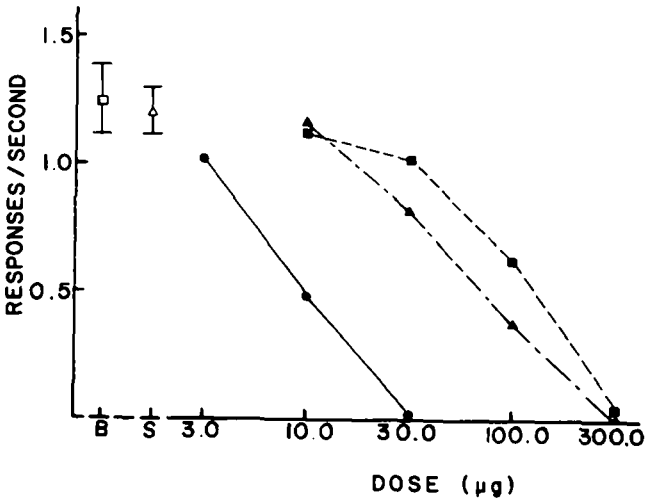

FIG. 1. Effects of intraventricular morphine (•), (D-Ala)^2-(des COOH-Norleucyl)^5-Enkephalin (▲), and (N$^\alpha$-methyl-Tyrosyl)^1-(des COOH-Norleucyl)^5-Enkephalin (■) on responding under a fixed-ratio 20 schedule of water reinforcement in rats. Each point is the mean of a single observation in each of 3 rats. The non-injection baseline (B, □) and the intraventricular saline control (S, △) points are the means (± SE) of 6 observations in each of the three rats. See methods for further details of the testing procedure and schedule.

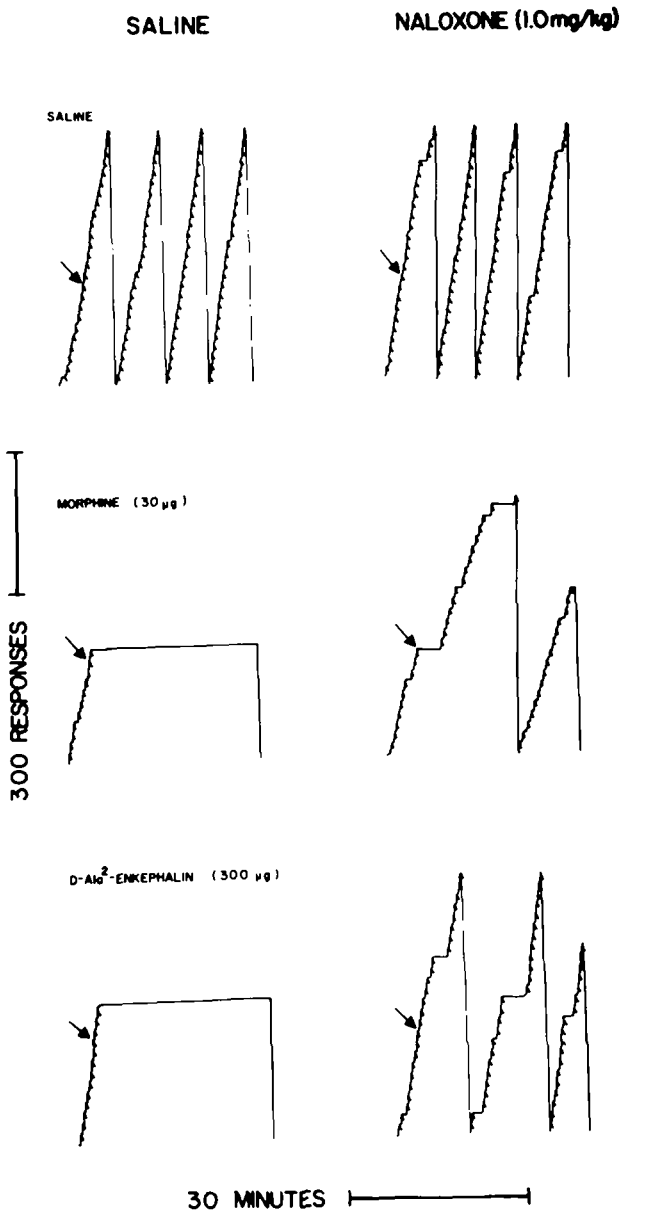

FIG. 2. Representative cumulative response records from one rat (No. 4) which demonstrates the effects of intraventricular morphine and (D-Ala)^2-(des COOH-Norleucyl)^5-Enkephalin on fixed-ratio 20 responding. Lever-press responses moved the response pen upward until 500 responses were emitted, at which point the pen automatically reset to the bottom of the paper. The pen also reset at the end of the session. Diagonal deflections of the response pens indicate presentations of the water-filled dipper (reinforcement). The recorder paper was advanced from left to right at a constant speed. Arrows indicate the times within the session when the intraventricular injections were delivered. Naloxone (1.0 mg/kg) was injected (SC) 15 min before the start of the session.

of morphine, relative behavioral potencies for the three compounds were determined on a molar basis, and morphine was about 5 times more potent than either of the two Enkephalin analogs. All three compounds appeared to have a rapid onset of action after intraventricular injection as shown for morphine and (D-Ala)^2-Enkephalin in Fig. 2. Intraventricular injection of (D-Ala)^2-Enkephalin (300 µg)

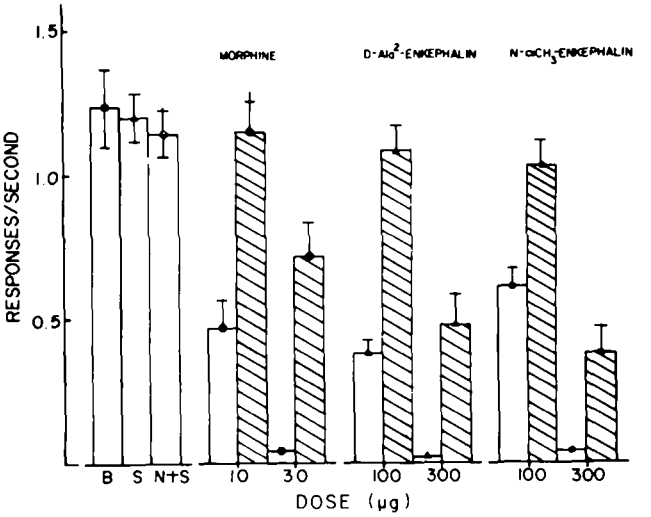

FIG. 3. Naloxone antagonism of the rate-decreasing effects of morphine, (D-Ala)^2-(des COOH-Norleucyl)^5-Enkephalin, and (N$^\alpha$-methyl-Tyrosyl)^1-(des COOH-Norleucyl)^5-Enkephalin on fixed-ratio 20 responding. Control data for non-injection baseline (B), saline injection control (S), and naloxone (1.0 mg/kg, SC) pretreated saline controls (S + N) represent the means of 2 observations in each of 3 rats (± SE). Data for morphine and the enkephalins are the means (± SE) of a single observation in each of 3 rats. Open bars represent the effect of intraventricular drug injection alone and the hatched bars are the effects of the intraventricularly administered drugs after naloxone pretreatment (1.0 mg/kg, SC; 15 min prior to the start of the session.

produced a rapid suppression of behavior which lasted for the remainder of the 30 min session. A similar rapid onset and long duration of action was also seen for morphine and the N$^\alpha$-methyl-Enkephalin.

Pretreatment with naloxone (1.0 mg/kg, SC) 15 min before the start of the session antagonized the rate decreasing effects of intraventricularly administered morphine and the two Enkephalin analogs (Figs. 2 and 3). Naloxone appeared to be equi-effective at two doses in antagonizing morphine and the Enkephalins. Under these limited conditions, it appeared that naloxone shifted the dose-effect curves to the right about 3 fold. Naloxone had no effect on responding at a dose of 1.0 mg/kg (SC) when compared to the non-pretreated saline controls (Figs. 2 and 3). In addition, the parentral administration of these peptides, had no behavioral effects when given at doses of up to 3 mg/kg.

DISCUSSION

The rate and pattern of FR responding in the present study was similar to that previously reported for FR20 responding in the pigeon [10] and the rat [30]. Intraventricular morphine injections produced dose-related decreases in FR responding at doses between 3 and 30 µg. The onset of the effect of morphine and the Enkephalin analogs appeared to be quite rapid following intraventricular injection. A similar rapid onset of action was reported by Cowan *et al.* [4] for the intraventricular injection of Methionine^5-Enkephalin in rats under a FR45 schedule of food reinforcement. Pickens and Thompson [25] have also reported a rapid onset of behavioral disruption produced by intravenous cocaine under a FR schedule of reinforcement in the rat. Compared to previously published dose-effect

curves for systemically administered morphine in the rat [30], intraventricular administration of morphine results in about a 100-fold increase in behavioral potency. The estimated ED_{50} for morphine-induced suppression of FR20 responding after intraventricular injection was about 0.4 mg/kg, compared to an estimated ED_{50} for systemic morphine of about 5.6 mg/kg [31]. Kutter *et al.* [18] has also reported a substantial increase in potency for morphine (900 fold) when morphine was injected intraventricularly to mice, compared to intravenous injection. In relation to the other opiate narcotics, morphine does not readily cross the blood-brain barrier [24] and the marked increase in morphine potency after intraventricular injection, compared to the other narcotics, is probably the result of by-passing the blood-brain barrier which is presumably the major limiting factor in morphine's distribution in the brain. Consistent with this hypothesis, Kutter *et al.* [18] observed less of an increase in potency after intraventricular injection of narcotics which more readily cross the blood-brain barrier, compared to morphine. Thus, the intraventricular injection route appears to provide an efficient means of studying drugs which are either not readily distributed to the brain or which are inactivated by the liver, lung or plasma enzymes.

$(D\text{-}Ala)^2$-Enkephalin and N^{α}-methyl-Enkephalin both produced decreases in operant behavior when injected intraventricularly. On a molar basis morphine was about 5 times more potent than either of the two enkephalin analogs. Neither the $(D\text{-}Ala)^2$-Enkephalin nor the N^{α}-methyl-Enkephalin produced any behavioral effects in rats when they were administered systemically in doses up to 3 mg/kg (J. Carney, unpublished observation). Cowan *et al.* [5] reported that Methionine^5-Enkephalin produced a transient suppression of FR45 responding at an intraventricular dose of 1,000 μg. The relatively brief duration of behavioral disruption produced by the Methionine^5-Enkephalin was probably due to the rapid enzymatic hydrolysis of the peptide by brain peptidases [12,18]. The two enkephalin analogs used in the present study were designed to be resistant to a range of peptidases (carboxypeptidases, aminopeptidases, aminodipeptidases, etc.). Using the guinea pig ileum bioassay, Day *et al.* [6] demonstrated that these two enkephalin analogs were resistant to destruction by brain peptidases for incubation periods up to 60 min at 37°C. Under these same incubation conditions Methionine^5-Enkephalin activity in the guinea-pig ileum was completely gone after 10 min of incubation [6]. Substitution of the des-COOH-Norleucine for Methionine in the 5-position resulted in a substantial increase in the duration of action of the enkephalin and indicates that at least one factor which limits the duration of the behavioral effects of the enkephalins is cleavage by a carboxypeptidase. The additional substitution of D-alanine for glycine in the 2-position of the pentapeptide, resulted in an increased duration of action, suggests that one other mechanism of enkephalin inactivation is by an amino dipeptidase. The addition of an α-methyl on the nitrogen of the tyrosine of enkephalin (position 1 of the pentapeptide) was designed to prevent hydrolysis by amino peptidases and it also had a long duration of action in the present study.

The data presented in the present study demonstrate that, like morphine, the enkephalins produce dose-related decreases in schedule-controlled responding which is reversed by naloxone. Both morphine and the enkephalins produce analgesia in animals [1, 6, 18, 25], which can be blocked by naloxone. Morphine tolerant-dependent rodents are cross-tolerant and cross-dependent on the enkephalins [3, 6, 33]. The effects of morphine and the enkephalins are blocked by specific narcotic antagonists (present data [7, 20, 21, 22]). Opiate narcotics and the enkephalins both inhibit the electrically induced contractions of the guinea pig ileum and the mouse vas deferens [6, 13, 15, 16, 25]. Both narcotics and enkephalins appear to interact with the high-affinity narcotic binding in brain [25,32]. It has been proposed that the naturally occurring enkephalins are the endogenous ligand for the opiate receptor through which morphine and all other narcotics act [15, 16, 30]. The present data extends the behavioral pharmacology of the enkephalins to include disruption of operant responding in the rat which appears related to an agonist effect of some narcotic receptor. It would be interesting to determine if animals trained to discriminate between intraventricular morphine and intraventricular saline would also identify the enkephalins as being morphine-like.

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