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Antagonism of Morphine-Induced Behavioral Suppression by Opiate Receptor Alkylators

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MESSING, R. B., P. S. PORTOGHESE, A. E. TAKEMORI AND S. B. SPARBER. *Antagonism of morphine-induced behavioral suppression by opiate receptor alkylators.* PHARMAC. BIOCHEM. BEHAV. 16(4) 621–626, 1982.—Experiments were conducted to test the *in vivo* opiate specificity and long-lasting effects of two non-equilibrium opiate antagonists: β-chlornaltrexamine (β-CNA) and the β-fumarate methyl ester derivative of naltrexone (β-FNA). β-CNA (2.5 or 5.0 μg, ICV) partially antagonized suppression of conditioned autoshaped behavior by morphine, when morphine was administered 48–72 hr after β-CNA. β-CNA had no effect on amphetamine-induced suppression of autoshaped responding, nor did it antagonize the suppression in rearing activity induced by either morphine or amphetamine. Similarly, β-FNA (5 mg/kg, IP) antagonized the suppression of conditioned behavior by morphine, for up to 48 hr, while having no effect on amphetamine-induced suppression of autoshaped responding, or on the suppression of rearing activity induced by morphine or amphetamine. Further peripherally administered β-FNA acts in the brain, since it antagonized analgesia following ICV morphine administration.

Amphetamine Autoshaped behavior Morphine Opiate receptor alkylators

THE nitrogen mustard analogue of naltrexone, β-chlornaltrexamine (β-CNA) and the β-fumarate methyl ester of naltrexone (β-FNA) appear to be potent nonequilibrium opiate antagonists. Thus, *in vitro*, β-CNA, in contrast to either chlorambucil or phenoxybenzamine, has been found to be a potent inhibitor of stereospecific ^{3}H-naloxone binding in mouse brain, even after extensive washing of tissue [2,11]. β-CNA also antagonized suppression of electrically-induced contractions in the guinea pig ileal longitudinal muscle by morphine but not by norepinephrine, again even after extensive washing of ileal tissue [3]. *In vivo*, after intracerebroventricular (ICV) administration, β-CNA antagonizes morphine analgesia for up to three days in the mouse [11]. More recently, β-FNA has been shown to be an irreversible narcotic antagonist in the guinea pig ileum [12]. β-FNA is a less chemically reactive, and hence may be a more selective antagonist than β-CNA because the aziridinium ion derived from the nitrogen mustard is more reactive than the fumarate group of β-FNA [12].

The present experiments were designed to investigate the opiate specificity and long-lasting effects of these agents *in vivo*, and also to find out if they affect behavior at doses which antagonize morphine analgesia. To do this the effects of β-CNA and β-FNA on performance of conditioned (autoshaped) behavior and upon exploratory (rearing) behavior were first measured. Both of these behaviors are suppressed by moderate to high doses of either morphine or amphetamine. The abilities of β-CNA and β-FNA to antagonize both morphine- and amphetamine-induced suppression of the autoshaped response and rearing were therefore also determined. This was done using doses of the alkylating agents and times after administration that were similar to those that we used in assaying antagonism of morphine analgesia by β-CNA and β-FNA.

METHOD

Animals

Male Holtzman rats, initially weighing 350–450 g, and maintained at 80–85% of initial body weight, were used in all experiments. Rats were maintained on a 12:12 hr light/dark schedule, with lights on at 7:00 a.m. Testing was done between 9:30 a.m. and 4:30 p.m.

Drugs

β-CNA and β-FNA were snythesized by methods previously described [11,12]. A stock solution of β-CNA (1 μg/μl) in 95% ethanol containing 0.05 M HCl was stored at −15°C. Immediately before injecting each animal, an aliquot of stock solution was evaporated to dryness under nitrogen, and the residue was taken up in 0.9% (w/v) NaCl. ICV injections were made in a volume of 5 μl, and given over an interval of 2.5 min. β-FNA was dissolved in 0.1 mM HCl on the day of

the experiment, and either 0.1 mM HCl or β-FNA solution (2 ml/kg) were injected IP. Morphine sulfate (Merck, Rahway NJ) and amphetamine sulfate (K. and K. Labs Inc., Plainview, NY) were injected in 0.9% NaCl vehicle (1 ml/kg) either SC or IP. All drug doses were calculated from the weight of the free base.

Cannula Implantation

Polytheylene cannulae (20 gauge) [13] were stereotaxically implanted into the left lateral ventricle of each animal. To verify cannulae placements, rats were injected ICV with 5 μl of fast green dye, brains were removed, and the presence of dye in the lateral ventricles and the piercing of the corpus callosum by the cannulae tips were visually determined.

Tail-Flick Assay

Analgesia was measured using the tail-flick method [4,7]. In experiments with β-CNA, baseline reaction times were determined for each rat, and β-CNA or vehicle were injected ICV immediately afterwards. Morphine (10 mg/kg, s.c.) was injected one hr later and tail-flick latencies were again determined 90 min after the β-CNA injections. If a rat did not perform a tail-flick response within 10 sec after stimulus onset, he was removed from the apparatus. Analgesia was defined as a reaction time greater than 3 standard deviations above the baseline mean.

In experiments with β-FNA, the ED_{50} for ICV morphine was determined. Four to five days later, rats were randomly reassigned to different groups and injected IP with 5 mg/kg of β-FNA. Forty-eight hr following these injections the ED_{50} for ICV morphine was again determined. Data from this experiment were analyzed by the parallel line assay method of Finney [5].

Autoshaped and Rearing Behavior

The autoshaping procedure and apparatus are described in detail elsewhere [8]. Briefly, rats were placed in a Skinner box containing a retractable lever. During each session a white light was turned on above the lever. Each lever presentation served as the exteroceptive stimulus for delivery of food pellets. Lever presentations were made according to a random interval (RI) schedule with the average interval between presentations set at 60 sec, and the maximum and minimum intervals between presentations 90 and 30 sec, respectively. The lever remained extended for 15 sec or until the rat contacted the lever. After 15 sec, or upon a lever contact, the lever was retracted and a food pellet delivered. Thirty lever presentations were made in each session. Rats were subjected to one 30 trial session per day on consecutive days. Data were recorded by a computer, and also by a cumulative recorder. Simultaneously with each lever extension, the stepping pen of the recorder was activated in 0.05 sec increments. The pen was reset by lever retraction, thus providing a graphic representation of latency to respond for each trial.

Each Skinner box was also provided with a metal strip, 7.5 cm wide and 12.5 cm above the floor, along each wall except that containing the lever. Each contact with the strip activated a microswitch, thus permitting recording of rearing (exploratory) activity.

β-CNA was administered to naive rats 90 min prior to their first daily exposure to the apparatus, and morphine or amphetamine was give 2–3 days later, 15 min prior to the test session. To make sure that morphine and amphetamine effects on behavior were not confounded with increasing experience of the apparatus, rats were retested one day following their last drug injections. Levels of operant and rearing responding returned to those observed 24 hr post β-CNA administration.

Because β-CNA had no effect on acquisition of autoshaped responding, it was decided to see if β-FNA had an effect of performance of an already acquired response, shortly after its administration. Therefore, rats were given β-FNA 90 min prior to their third daily exposure to the apparatus, and morphine or amphetamine were injected 1–2 days later, 15 min prior to the test session. Also, based on previous results, the morphine dose was decreased slightly and the amphetamine dose was increased in this experiment to influence operant responding in the control group in the appropriate directions.

Data Analysis

Student's *t*-tests (two-tailed) were used to compare drug groups with vehicle controls. Since animals were used sequentially over several consecutive days, *t*-tests (one-tailed) for paired observations were used when data from a group of animals obtained on one day were compared with data from the same group obtained on a different day.

RESULTS

β-CNA Antagonism of Morphine Analgesia

An initial experiment was done to see if ICV β-CNA antagonizes morphine analgesia in rats as well as mice, and to find the effective dose range. Preliminary work established that the antagonism of morphine analgesia following β-CNA is entirely gone one week after ICV injection. Therefore, rats were used twice at an interval of one week. No differences were found in tail-flick latencies after morphine, between rats given any dose of β-CNA for the first time and rats previously given another dose of β-CNA.

All rats given 10 mg/kg of morphine SC 1 hr after ICV saline had tail-flick latencies in excess of 10 sec, following morphine administration. Fig. 1 shows the dose-response relationship of β-CNA in antagonizing morphine analgesia. The left side shows the increase in tail flick latencies following morphine and various doses of β-CNA. The right side shows the proportion of rats exhibiting an analgesic response after morphine and β-CNA administration (i.e., with reaction times greater than 3 standard deviations above the baseline mean). It is apparent by either measure that the degree of antagonism increases with the dose of β-CNA. However, even with 10 μg of β-CNA there is a significant increase in the mean tail-flick latency from the baseline mean, 30 min after morphine ($t=3.19$, 1-tail matched pairs *t*-test, $df=6$, $p<0.01$). Some signs of hyperactivity (e.g. jumping) were also observed in rats given 10 μg of β-CNA, but were not observed in rats given lower doses.

Effects of β-CNA on Autoshaped and Rearing Behavior

Based on the results of the previous experiment, the effects of 1.0, 2.5 and 5.0 μg of ICV β-CNA on rearing and acquisition of conditioned (autoshaped) behavior were investigated. Rats were injected with saline or β-CNA 90 min prior to being introduced to the behavioral apparatus for the

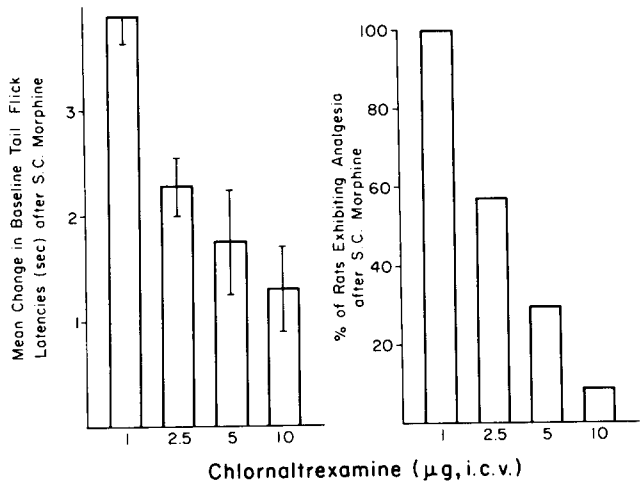

FIG. 1. Antagonism of morphine-induced analgesia by β-CNA. Baseline latencies were obtained in the tail-flick to thermal stimulus test. Rats were injected ICV with saline or β-CNA immediately afterwards, and SC with 10 mg/kg morphine one hr later. Tail-flick latencies were again obtained 30 min after morphine injections. Baseline latencies in sec were as follows (Means±SEM): Saline: 3.06±0.14; 1 μg β-CNA: 3.47±0.18; 2.5 μg β-CNA: 3.56±0.29; 5.0 μg β-CNA: 3.27±0.22; 10.0 μg β-CNA: 3.56±0.29. All rats in the saline group had latencies in excess of 10 sec following morphine administration. N= 7 rats/group.

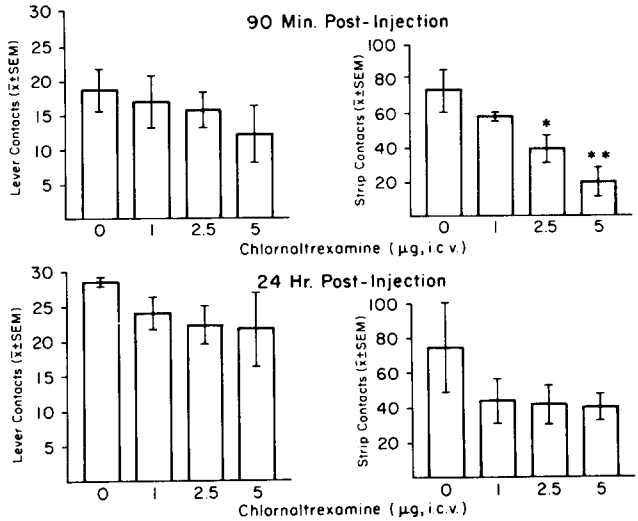

FIG. 2. Effect of β-CNA on rearing activity and acquisition of an autoshaped response. Rats were injected ICV with β-CNA or saline and tested for acquisition of a conditioned (autoshaped) response and rearing activity 90 min and 24 hr later. Unconditioned responding was measured by number of contacts made with a metal strip placed above the floor of the behavioral apparatus. N= 4–6 rats/group. *$p<0.05$, **$p<0.01$ compared to saline injected group (two-tailed t-test).

first time. One day later, rats were exposed to another 30-trial training session. Figure 2 (left) shows that β-CNA had no significant effect on response acquisition (i.e., on whether or not rats made a lever contact during each 15 sec period of lever extension) either 90 min or 24 hr post-injection. In contrast (Fig. 2, right side) 2.5 or 5.0 μg of β-CNA significantly suppressed rearing (strip contacts) 90 min after administration.

Forty-eight hr after being given β-CNA, one-half of the rata were injected with morphine and the other half with amphetamine, IP 15 min before being placed in the behavioral apparatus. The next day (72 hr post β-CNA administration) rats were injected with the drug (morphine or amphetamine) not given on the previous day, and then retested. No differences were apparant in data from rats injected with a given drug 2 or 3 days after β-CNA, so data for each drug, obtained 2 or 3 days following β-CNA administration, were pooled.

Both morphine and amphetamine significantly lowered the number of lever contacts in rats given ICV saline (Fig. 3, left side) when compared to data obtained 24 hr after ICV saline injections (Fig. 2, lower left) (all p's <0.001, one-tailed by matched pairs t-tests). β-CNA (2.5 or 5.0 μg) significantly antagonized the suppressant action of morphine, but not amphetamine (Fig. 3, left side). Thus, the effects of β-CNA show opiate specificity.

Morphine and amphetamine also significantly lowered the number of rearing responses in rats given ICV saline (Fig. 3, right side) when compared to data obtained 24 hr after ICV injections (Fig. 2, lower right) ($p<0.025$, one-tailed, by matched pairs t-test). β-CNA had no significant effect on the number of strip contacts made by rats given either morphine or amphetamine, in comparison to the ICV saline control group given these drugs (Fig. 3, right side). In agreement with this finding, morphine significantly suppressed rearing

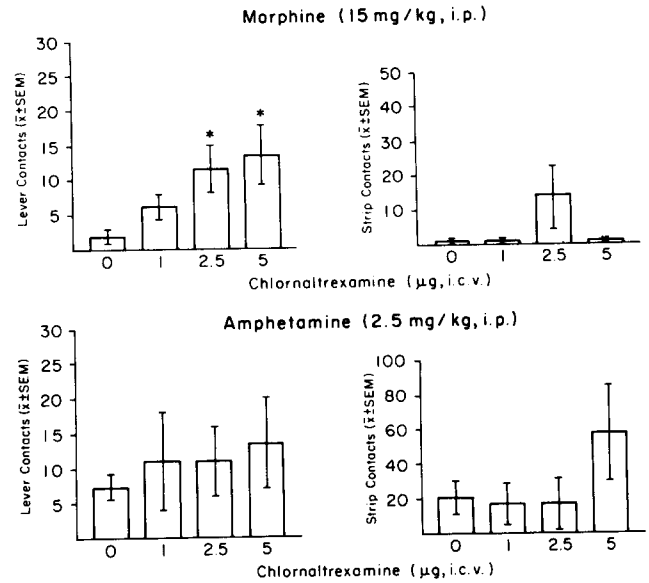

FIG. 3. Antagonism of morphine-induced suppression of conditioned responding by β-CNA. Rats were given β-CNA 2–3 days before testing and trained to contact a lever by an autoshaping procedure. Fifteen min prior to testing, rats were administered morphine or amphetamine and tested for conditioned responding (number of lever contacts in a 30-trial session) and exploratory (rearing) activity (number of strip contacts). N=4–6 rats/group. *$p<0.05$ compared to saline-injected group (two-tailed t-test).

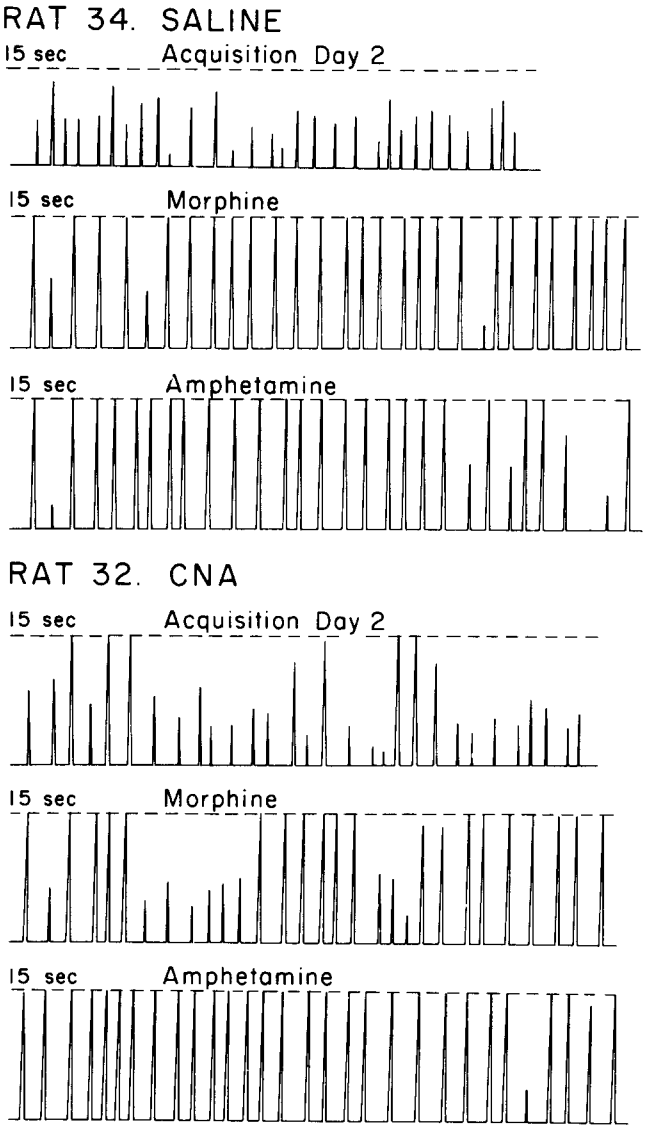

FIG. 4. Data from cumulative records of individual rats illustrating antagonism of morphine- but not amphetamine-induced suppression of conditioned responding by ICV β-CNA. Rats were trained by an autoshaping procedure to contact a lever and injected IP with morphine or amphetamine 15 min prior to behavioral testing. Each pen deflection represents the number of seconds the lever remained extended on each trial of the 30-trial session. Pen deflections less than maximum height indicate lever contacts by rat. Each strip represents one 30-trial session. Lever presentations were made according to an RI 60 sec schedule. The lever was retracted when the rat contacted it or after 15 sec.

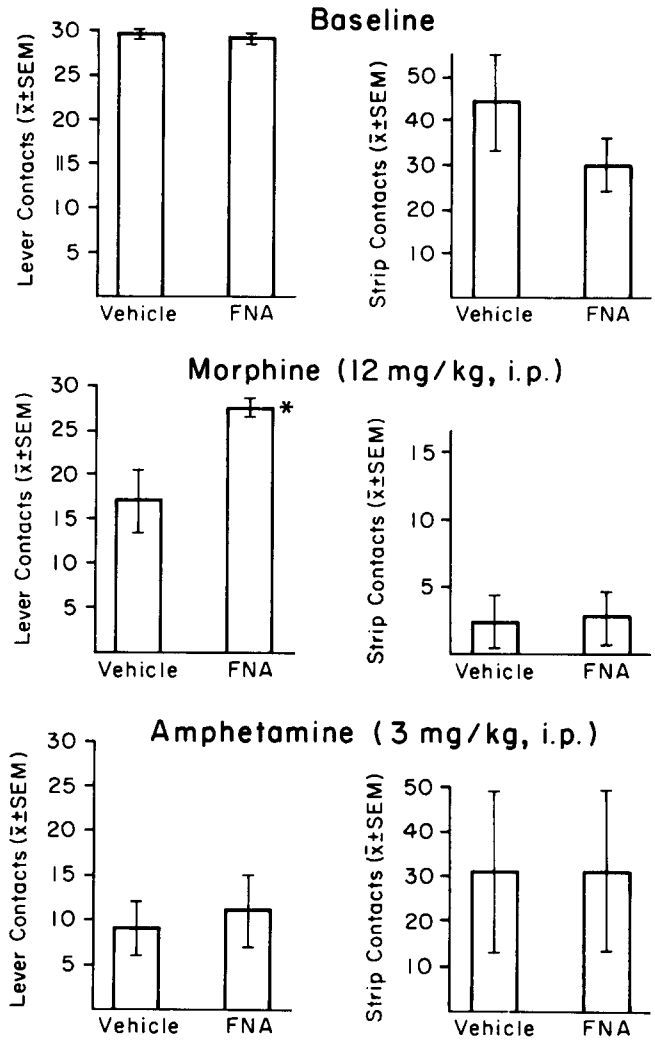

FIG. 5. Effects of β-FNA on morphine- and amphetamine-induced suppression of performance of an autoshaped response. Rats trained to contact a lever by an autoshaping procedure were administered saline or β-FNA. Ninety min later baseline conditioned performance (number of lever contacts in the 30-trial session) and rearing activity (strip contacts) were measured. One-two days later rats were given morphine or amphetamine. Fifteen min later conditioned responding and rearing activity were again measured. N=7–8 rats/group. *$p<0.01$ compared to vehicle-injected group (two-tailed t-test).

activity in all groups given β-CNA, when compared to rearing activity 24 hr after ICV injections (all p's <0.03, one-tailed, by matched pairs t-tests). Thus, β-CNA did not antagonize morphine effects on rearing activity. There may, however, be interactive effects of β-CNA and amphetamine on locomotor activity. β-CNA did not change rearing activity of amphetamine-injected rats when these rats are compared to amphetamine-injected rats not given β-CNA. However, amphetamine failed to significantly reduce rearing activity (by matched pairs t-test) in groups given 1.0 or 5.0 μg of β-CNA compared to rearing 24 hr after β-CNA (see Fig. 2, lower right and Fig. 3, lower right). Thus the effects of β-CNA on rearing activity in rats given amphetamine are difficult to interpret.

Data from the cumulative records of individual rats are shown in Fig. 4. These rats were chosen because the data obtained from them are closer to the mean values shown in Fig. 3 than data from other rats. These results graphically illustrate the suppression of conditioned responding by morphine and amphetamine in a single rat given an ICV saline injection. Thus, rat 34 made 30 lever contacts on Acquisition Day 2, 3 lever presses after morphine administration and 5 after amphetamine. In contrast, autoshaped behavior is

only partially suppressed in the rat given 5 μg of β-CNA, while the suppressant effect of amphetamine is unimpaired. Thus, rat 32 made 25 lever contacts on Acquisition Day 2, 12 after morphine administration and 2 after amphetamine.

Effects of β-FNA on Autoshape and Rearing Behavior and Analgesia

In the final experiment some behavioral and opiate-specific effects of β-FNA were investigated. Because β-FNA is a less reactive compound than β-CNA, it was of interest to see if it has activity after peripheral administration. Since β-CNA had no effect on acquisition, the effect of an irreversible opiate antagonist on performance of an already acquired conditioned response were investigated. Because of the limited quantity of drug available, it was possible to investigate only one dose (5 mg/kg).

Rats were given 2 autoshaping sessions on consecutive days. On the day following the second session, they were injected with vehicle or β-FNA IP, and tested 90 min later. Figure 5 (top) shows that β-FNA has no significant effect on performance of an autoshaped response or on rearing.

The next day (24 hr post β-FNA administration) one-half of the rats were injected IP with morphine and the other half with amphetamine, and conditioned responding and rearing were measured 15 min later. On the second day following β-FNA administration, rats were injected with the drug not given on the previous day. Since no differences were observed in data obtained from rats injected with a given drug one or two days after β-FNA administration, data from rats given each drug were pooled across days. The middle panel of Fig. 5 shows that β-FNA reversed the suppression of the lever contact response induced by morphine, but did not antagonize the morphine-induced suppression of rearing behavior. In contrast, the lower panel of Fig. 5 illustrates the lack of effect of β-FNA in antagonizing suppression of conditioned responding by amphetamine. In this experiment, amphetamine failed to significantly reduce rearing, and β-FNA did not alter rearing in rats given amphetamine.

Figure 6 presents data from individual rats. Again, rats were chosen because the data obtained from them are close to mean values. It can be seen that both morphine and amphetamine suppressed the lever contact response in a vehicle-injected rat. Rat 2 made 28 lever contacts on Day 3, 10 following the morphine injections and 2 following amphetamine. In the rat injected with β-FNA, only amphetamine suppressed conditioned responding. Rat 4 contacted the lever 28 times on Day 3, and 28 times after morphine administration. However, only 2 lever contacts were made after the amphetamine injection.

Finally, we determined whether peripherally administered β-FNA acts in the brain, by assessing its ability to antagonize the analgesic effects of ICV morphine in the tail-flick test. Table 1 shows that β-FNA significantly decreased morphine-induced analgesia 48 hr after administration (i.e., there is no overlap in the 95% confidence limits of the ED_{50}'s for morphine determined in the presence or absence of β-FNA). These results have since been confirmed in a more extensive series of experiments [14].

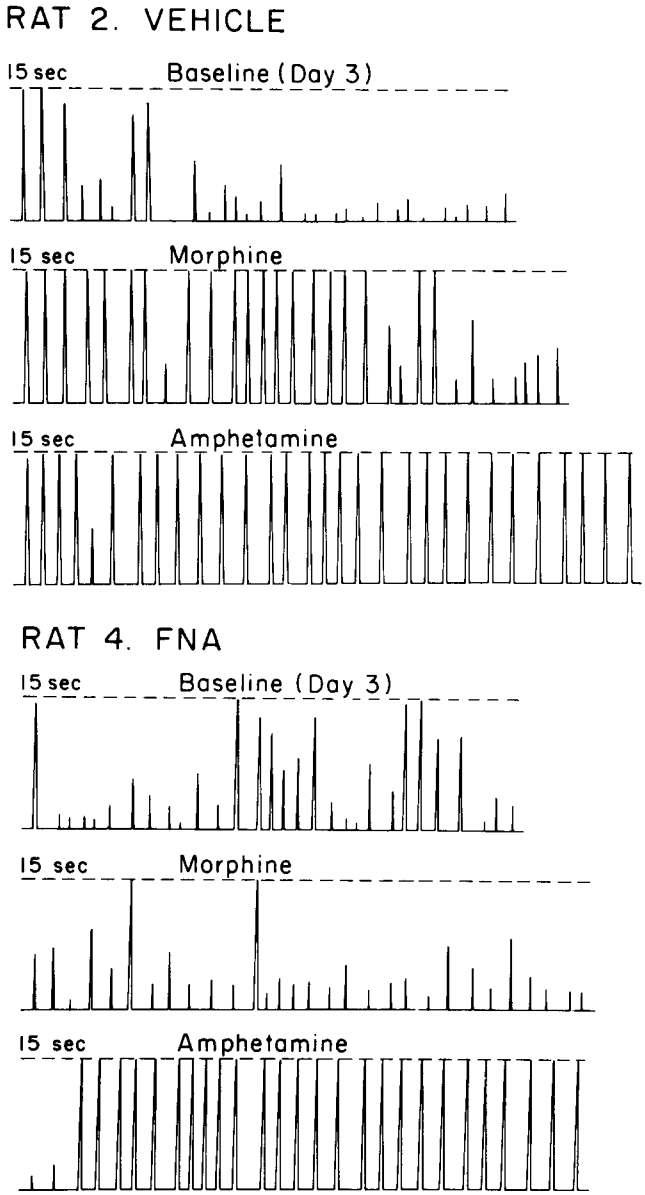

FIG. 6. Data from cumulative records of individual rats illustrating antagonism of morphine- but not amphetamine-induced suppression of conditioned responding by β-FNA. Baseline performance in an autoshaped task in which rats learned to contact a lever was measured 90 min after IP injection of vehicle of β-FNA. One-two days later rats were injected IP with morphine or amphetamine and conditioned responding was measured 15 min post-injection. Each pen deflection represents the number of seconds the lever remained extended on each trial of the 30-trial session. Pen deflections less than maximum height indicate lever contacts by rat. Each strip represents one 30-trial session. Lever presentation was made according to an RI 60 sec schedule. The lever was retracted when the rat contacted it or after 15 sec.

DISCUSSION

These results demonstrate that both β-CNA and β-FNA partially antagonize morphine analgesia and morphine-induced suppression of conditioned responding in the rat.

TABLE 1

INCREASED ANALGESIC ED_{50} OF ICV MORPHINE AFTER PERIPHERAL β-FNA ADMINISTRATION

Morphine Sulfate (μg ICV)	% Rats Exhibiting Analgesia	ED_{50} (μg)
	Control	
1.25	16.7	
2.50	50.0	2.30 (1.75–2.93)
5.00	100.0	
	β-FNA	
5.0	50.0	
10.0	66.7	5.10 (4.54–5.67)
40.0	100.0	

The ED_{50} for ICV morphine sulfate was determined 7–9 days after cannulae implantations. Four to five days later, rats were injected with β-FNA (5 mg/kg, IP) and ED_{50}'s were again determined 48 hrs following FNA injections. N=6 rats/group. Values in parentheses are 95% confidence limits for the ED_{50}'s.

The morphine-antagonistic effects of β-CNA were shown to last for up to three days. The effectiveness of β-FNA, a more specific compound [12], in antagonizing morphine-induced analgesia and suppression of conditioned behavior, 24–48 hrs after systemic administration, was also demonstrated. Antagonism of morphine's effects after systemic administration of the long acting opiate antagonist naloxazone has been previously reported [10]. However, far higher doses of this compound (75–200 mg/kg) were required than the dose of β-FNA used in this research. These data also indicate that both β-FNA and β-CNA have at least some degree of specificity for opiate receptors *in vivo*, since they did not significantly modify amphetamine-induced suppression of conditioned behavior, when rats give β-CNA or β-FNA and amphetamine were compared to rats treated with vehicle and amphetamine.

In contrast to the antagonism of morphine-induced suppression of autoshaped behavior by β-CNA and β-FNA, both compounds failed to modify exploratory activity after morphine administration. One explanation for this is that these compounds suppress rearing, and this suppression masks any antagonism of morphine-induced suppression of activity. Other investigators [1,9] have found, for instance, that both naloxone and naltrexone reduce locomotor activity in the rat under certain conditions. This explanation appears inadequate, however. We found only a small locomotor suppressant effect of β-CNA, and no significant effect of β-FNA on uncondidtioned exploratory activity. Thus, an alternative explanation may be simply that the reduction in rearing after morphine administration is mediated by a receptor population which is relatively insensitive to these opiate antagonists.

In summary, β-CNA and β-FNA both appear to be relatively non-toxic (as measured by effects on operant and rearing behavior), long-lasting, specific opiate antagonists *in vivo*. Furthermore, the ability of β-FNA to exert long-lasting opiate-antagonistic effects after systemic administration of a relatively small dose, points to its potential therapeutic usefulness as an ultralong-acting narcotic antagonist.

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