Antianalgesic Action of Dynorphin A Mediated by Spinal Cholecystokinin (44361)

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Abstract. **Previous work indicates that the antianalgesic action of pentobarbital and neurotensin administered intracerebroventricularly in mice arises from activation of a descending system to release cholecystokinin (CCK) in the spinal cord where CCK is known to antagonize morphine analgesia. Spinal dynorphin, like CCK, has an antianalgesic action against intrathecally administered morphine. This dynorphin action is indirect; even though it is initiated in the spinal cord, it requires the involvement of an ascending pathway to the brain and a descending pathway to the spinal cord where an antianalgesic mediator works. The aim of the present investigation was to determine if the antianalgesic action of intrathecal dynorphin A involved spinal CCK. All drugs were administered intrathecally to mice in the tail flick test. Morphine analgesia was inhibited by dynorphin as shown by a rightward shift of the morphine dose-response curve. The effect of dynorphin was eliminated by administration of the CCK receptor antagonists lorglumide and PD135 158. One hour pretreatment with CCK antiserum also eliminated the action of dynorphin. On the other hand, the antianalgesic action of CCK was not affected by dynorphin antiserum. Thus, CCK did not release dynorphin. Both CCK and dynorphin were antianalgesic against DSLET but not DPDPE, δ_2 and δ_1 opioid receptor peptide agonists, respectively. The results suggest that the antianalgesic action of dynorphin occurred through an indirect mechanism ultimately dependent on the action of spinal CCK.** [P.S.E.B.M. 1999, Vol 220]

The intracerebroventricular, i.c.v., administration of pentobarbital (1, 2) produces an antianalgesic action that is mediated by spinal cholecystokinin, CCK. Antianalgesic action is defined as the ability of an agent to antagonize the analgesic action of morphine by acting at a site different from where morphine acts. Spinal CCK is the proximal agent involved in pentobarbital antagonism of morphine analgesia. Spinal release of CCK is also involved in the antianalgesic action of neurotensin given i.c.v. in rats (3–5) and mice (Holmes BB *et al.*, unpublished data). These latter studies were preceded by the knowledge that spinal CCK antagonizes the analgesic action of morphine (6–9).

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The opioid peptide dynorphin A (1–17), Dyn, given at a very small dose (10 pg or 5 femtomoles) intrathecally, i.t., in mice also produces an antianalgesic action against morphine (10, 11). This antianalgesic action has been shown to be indirect in that spinal transection eliminates it (11). In barbital-anesthetized mice, 1 hr after spinal transection, the ability of i.t. morphine to inhibit the tail flick response is unchanged. However, i.t. Dyn is no longer able to inhibit this morphine action. Another indication for the role of the brain is that flumazenil, a benzodiazepine receptor antagonist, given i.c.v., inhibits the antianalgesic action of i.t. Dyn (12). Thus, i.t. Dyn activates an ascending system to the brain (step 1, Fig. 1), which in turn activates a system within the brain (step 2) that stimulates a descending system to the spinal cord to antagonize the effect of i.t. morphine in the tail flick test (step 3). The sequential link between Dyn and CCK systems is suggested by the fact that the antianalgesic action of i.c.v. pentobarbital, which involves release of spinal CCK but not Dyn, is inhibited by i.c.v. flumazenil (1, 2). The purpose of the present investigation was to determine if the antianalgesic action of i.t. Dyn, like that of pentobarbital and neurotensin, is mediated by spinal CCK. Studies were designed to determine whether i.t. administration of CCK

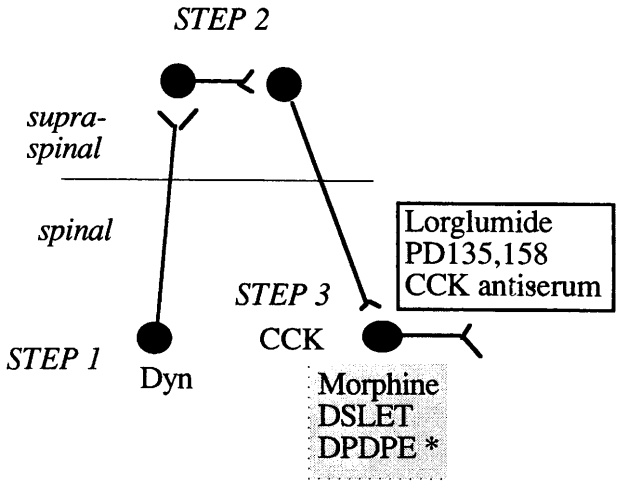

Figure 1. Antianalgesic action of Dyn given i.t. against morphine proposed to occur through antianalgesic action of spinal CCK. In step 1 Dyn given i.t. acts in the spinal cord to activate an ascending system to the brain. In step 2, the stimulus acts in the brain (an action that can be inhibited by flumazenil, a benzodiazepine receptor antagonist). In turn, in step 3, the stimulus transmitted through a descending system releases spinal CCK. CCK antagonizes the action of morphine in the tail flick test. In the present study this scheme is evaluated by administration of the CCK receptor antagonists lorglumide and PD135 158 and CCK_B antiserum. * CCK is known not to antagonize DPDPE, a δ_1 opioid receptor agonist action so that ability of Dyn and CCK to antagonize DPDPE and DSLET, a δ_2 receptor agonist, is compared.

receptor antagonists would inhibit the antianalgesic action of i.t. Dyn. An antiserum against CCK8 was also given i.t. for the same purpose.

Materials and Methods

Animals and Measurement of Antinociception. Male CD-1 mice weighing 25–30 g were obtained from Charles Rivers (Wilmington, MA). Each animal was used for only one experiment. In the radiant heat tail flick test for antinociception (13), the predrug control tail flick latency was determined to be 2–4 sec. Two tail flick test trials were conducted prior to the administration of drugs, and the average was employed as the predrug time. A cut-off time set at 10 sec was used as the maximal antinociceptive response. The percentage maximum possible effect (% MPE) was calculated (14) by the formula:

$$\% \text{ MPE} = \frac{[(\text{Postdrug})-(\text{Predrug})] \times 100}{(10-\text{Predrug})}$$

Drug Administration. All drugs were administered i.t. in a volume of 5 μl by the method of Hylden and Wilcox (15) 5 min before the tail flick test (unless stated otherwise). Appropriate vehicle solutions were administered when a drug was not given. Morphine and the CCK antagonists were dissolved in a 0.9% saline solution and Dyn, DPDPE, and DSLET were dissolved in a 0.9% saline solution containing 0.01% Triton X-100. Doses and times of administration of drugs are given with each experiment and based on previous publications (Holmes *et al.*, unpublished data, 10–12). All studies were done in compliance with the Institutional Animal Care and Use Committee (Animal Studies Subcommittee).

Statistical Analysis. For the morphine dose response data, the percentage MPE values were plotted versus the log dose for i.t. morphine (as altered by Dyn and then CCK antagonist or CCK8 antibody treatment), and ED_{50} values were derived and compared by a computerized version of the method of Litchfield and Wilcoxon (16) as described by Dewey *et al.* (14). Analysis of single-dose experiments that involved a comparison between two groups was by Student's *t* test. Those involving more than two groups were first analyzed by analysis of variance and, significant differences were analyzed further by comparing all the groups to each other using Neuman-Keul's test (17). In the latter situation, only the results for the main comparisons are given even though all possible comparisons were made. A $P \leq 0.05$ was taken to indicate a significant difference between groups in all of these analyses.

Source of Drug. The drugs and commercial sources were as follows: morphine sulfate.$5H_2O$ (Mallinckrodt Chemical Works, St. Louis, MO); Dynorphin A (1–17); and DSLET (Peninsula Laboratories, Inc., Belmont, CA). The CCK_A receptor antagonist lorglumide sodium salt (7, 18)) and the CCK_B receptor antagonist PD135 158 N-methyl-D-glucamine salt(4-{[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[[1,7,7-trimethylbicyclo [2,2,1]hept-2-yl)oxy] carbonyl] amino] propyl]amino]-1-phenylethyl]amino-4-oxo-[1*S*-1α,2β[S*(S*)]4α]}-butanoate N-methyl -D-glucamine (bicyclo system 1*S*-endo) (19) were obtained from Research Biochemicals International (Natick, MA). DPDPE was obtained from Bachem Inc. (Torrance, CA) or Sigma (St. Louis, MO). CCK8 antiserum from Chemicon International Inc. (Temecula, CA) and Dyn antiserum produced in rabbits in our laboratory were as used previously (Holmes *et al.*, unpublished data; 2, 10, 20–22). The doses of the drugs, given with the experiments, were for the forms stated above.

Results

The Effect of i.t. Administration of CCK Receptor Antagonists on the Antianalgesic Action of i.t. Dyn. The antianalgesic action of i.t. Dyn was demonstrated by its ability to antagonize the analgesic action of i.t morphine, Fig. 2(A). This action was eliminated in a dose-dependent fashion by i.t. administration of lorglumide, a CCK_A receptor antagonist. Similarly, the antianalgesic action of Dyn was eliminated by i.t. administration of the CCK_B receptor antagonist PD135 158 in a dose-dependent manner (Fig. 2B).

Next, dose response curves were examined for i.t. morphine alone and in the presence of fixed doses of Dyn and lorglumide (Fig. 3). Morphine alone had an ED_{50} (95% confidence interval) value of 0.59(0.36–0.96) μg, (Table I). Co-administration of Dyn i.t. shifted the morphine dose response curve to the right to yield an ED_{50} of 3.00(1.00–9.10) μg. The administration of lorglumide (1 μg) i.t. shifted the morphine curve back to the left to give an ED_{50} value of 0.50(0.21–1.20), μg which was near that for the morphine control.

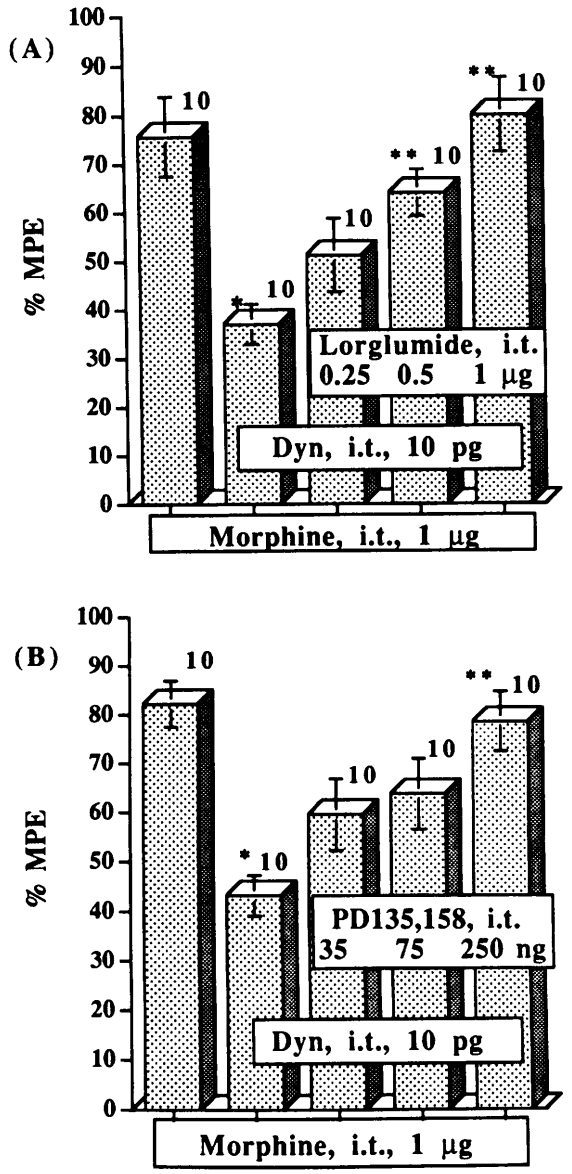

Figure 2. Dose response effect for (panel A) lorglumide and (panel B) PD135 158 inhibition of the antianalgesic action of Dyn. All the drugs were administered at the stated doses i.t. 5 min before the tail flick test. The bar represents the mean percentage MPE; the vertical line at the top of the bar is the SEM, and the number next to this line is the number of mice in the group. A single (*) and double asterisk (**) indicate significant differences from the morphine alone and morphine + Dyn groups, respectively, by ANOVA followed by Newman-Keuls' test ($P \leq 0.05$).

A similar set of experiments (Fig. 4) demonstrated that the rightward shift produced by i.t. Dyn was also antagonized by co-administration of 100 ng of PD135 158. The dose response curve for i.t. morphine and i.t. morphine in the presence of i.t. Dyn are the same curves as in the previous figure. The PD135 158 treatment shifted the curve back to give an ED_{50} value of 0.52(0.26–1.00), μg which was not different from that for morphine alone.

The Effect of i.t. Administration of CCK Antiserum on the Antianalgesic Action of i.t. Dyn. The antianalgesic action of i.t. Dyn was eliminated by the administration of a 1:1000 dilution of CCK8 antiserum (Fig. 5). The dose response curves for i.t. morphine alone and in the presence of Dyn are from before. A 1-hr pretreatment with the CCK8 antiserum shifted the dose response curve of i.t. morphine in the presence of Dyn to give an ED_{50} value no different from that for the morphine alone (Table I).

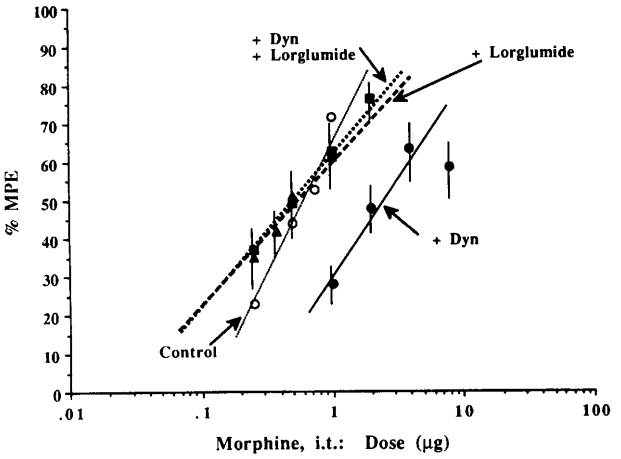

Figure 3. Dose response relationship for i.t. morphine alone (open circle) and in the presence of Dyn, i.t., 10 pg (black circle), lorglumide, i.t., 1 μg (black triangle), and Dyn + lorglumide (black square).

Table I. ED_{50} (95% Confidence Interval) Values for Morphine Given i.t. under Various Treatment Conditions

Treatment	ED_{50} (95% CI) μg
Control	0.59 (0.36–0.96)
+Dyn, i.t., 10 pg	3.00[a] (1.00–9.10)
+Dyn, i.t., 10 pg +Lorglumide, i.t., 1 μg	0.50 (0.21–1.20)
+Dyn, i.t., 10 pg +PD135158, i.t., 100 ng	0.52 (0.26–1.00)
+Dyn, i.t., 10 pg +CCK8 AS, i.t., 1:1000	0.29 (0.12–0.71)

[a] ED_{50} value of this group was significantly different from that of the other groups, $P < 0.05$ using statistical procedure as described in Methods, whereas the ED_{50} values of the other treated groups were not significantly different from the control group, $P > 0.05$.

In an experiment in which a single dose of i.t. morphine was used, a 1-hr pretreatment with Dyn antiserum had no effect on the antianalgesic action of i.t. CCK8 s (data not given). The Dyn antiserum inhibited the antianalgesic action of i.t. Dyn.

Results for Some Additional Controls. Table II provides information about additional controls that were missing in some of the above experiments. Triton X-100 treatment had no effect on the control response. Lorglumide treatment by itself and combined with Dyn had no effect. Similar results were found with PD135 158 alone and with Dyn. CCK8 antiserum by itself and with Dyn had no effect. These negative findings indicate that neither spinal CCK nor Dyn seem to be tonically released. Tonic release of either would be antianalgesic, and inhibition of such antianalgesic action would be expected to produce analgesia.

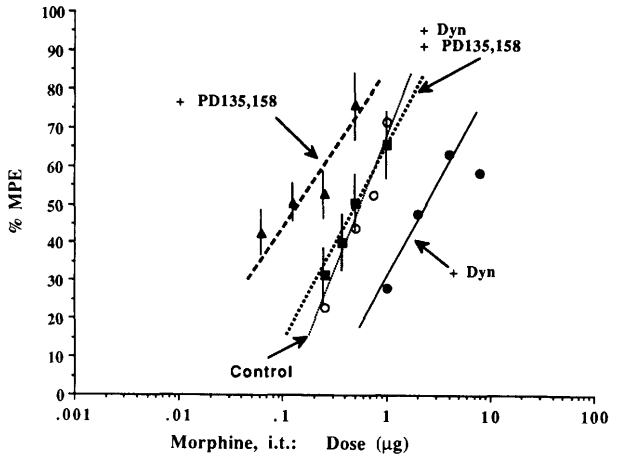

Figure 4. Dose response relationship for i.t. morphine alone (open circle) and with Dyn (black circle) (these data are from Figure 3) in the presence of PD135,158, i.t., 100 ng (black triangle) and Dyn + PD135 158 (black square).

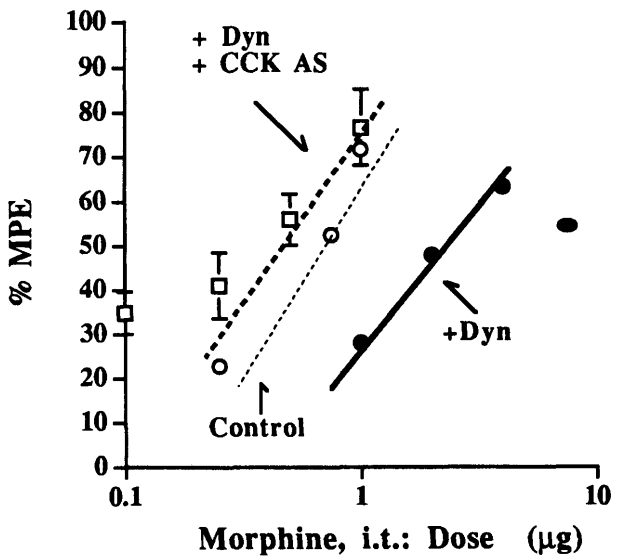

Figure 5. Dose response relationship for i.t. morphine alone (open circle) in the presence of Dyn (black cirle) (these data are from Figure 3) and Dyn + CCK_8 antiserum, diluted 1/1000 and given 1 hr before the tail flick test (open square).

Table II. The Tail Flick Response for Various Treatments Given i.t. (n = 10 for all groups)

Treatment	% MPE (SEM)
0.01% Triton X-100, 5 μl, 5 min	3.2 (1.5)
Lorglumide, 1 μg, 5 min	5.9 (2.2)
Lorglumide+Dyn, 10 pg, 5 min	1.9 (2.8)
PD135,158, 100 ng, 5 min	7.1 (2.5)
PD135,158+Dyn, 10 pg, 5 min	3.2 (2.4)
CCK8 AS, 1:1000, 1 hr[a]	−1.0 (5.9)
CCK8 AS+Dyn, 10 pg, 5 min	9.9 (5.2)

[a] Data for CCK8 AS by itself is from a previous publication (2).

A Comparison of the Antianalgesic Actions of CCK8s and Dyn Against i.t. DPDPE- and DSLET-Induced Analgesia. In Figure 6, the analgesic action of i.t. DPDPE, a peptide agonist relatively selective for the δ_1 opioid receptor subtype, was not affected by either i.t. CCK8s or Dyn administration. On the other hand, the analgesic action of i.t. DSLET, a peptide relatively selective for the δ_2 opioid receptor subtype, was inhibited by administration of both CCK8s and Dyn. CCK8s given at a 1-ng dose effectively inhibited DSLET-induced analgesia whereas even a 5-ng dose of CCK8s did not alter the analgesic action of DPDPE. These data demonstrated a parallelism between the antianalgesic actions of CCK8s and Dyn.

Discussion

The results indicate that the features of the antianalgesic action of Dyn match those expected if CCK were the mediator. The antianalgesic action of i.t. Dyn was inhibited by i.t. administration of the CCK receptor antagonists lorglumide and PD135 158. Previously these two agents administered i.t. were shown to inhibit the antianalgesic action of i.t. CCK8s in mice (2). The doses of each agent shown previously to inhibit the antianalgesic action of CCK8s given i.t. and released spinally by i.c.v. pentobarbital (2) and neurotensin (Holmes BB *et al.*, unpublished data) were effective in producing about the same magnitude of inhibition of the antianalgesic action of i.t. Dyn. The antianalgesic action of Dyn was also inhibited by the administration of CCK8 antiserum (which has been shown previously to inhibit the antianalgesic action of CCK8s (2, Holmes BB *et al.*, unpublished data)).

Furthermore, a parallel selectivity in the antianalgesic actions of Dyn and CCK was observed. Neither Dyn nor CCK8s given i.t. inhibited i.t. DPDPE analgesia. Previous reports demonstrate that spinal CCK antagonizes the μ agonist analgesic action of morphine but not the δ agonist action of DPDPE (23). However, both Dyn and CCK inhibited the action of i.t. DSLET. The results indicate that the same selectivity of action occurred for i.t. Dyn as for CCK8s, a finding consistent with the concept that Dyn works through CCK. The evidence in the literature (24) is not sufficient to explain why DSLET, but not DPDPE-induced analgesia, is antagonized by CCK8s. DSLET is relatively free of κ agonist action, but the possibility that it might be acting through μ receptors cannot be excluded even though its 30-fold-higher affinity for δ over μ receptors (25, 26) might suggest otherwise. Exactly how CCK antagonizes opioid-induced analgesia selectively is not known even though some mechanisms have been proposed (24, 27–29).

The reverse possibility that CCK might work through Dyn was also considered because the evidence provided in the present study up to this point could be explained by either possibility. The finding that the administration of Dyn antiserum i.t. did not affect i.t. CCK-induced antianalgesia eliminated the reverse possibility. Further evidence against the reverse possibility comes from earlier work. The antianalgesic action of i.c.v. pentobarbital involves spinal CCK

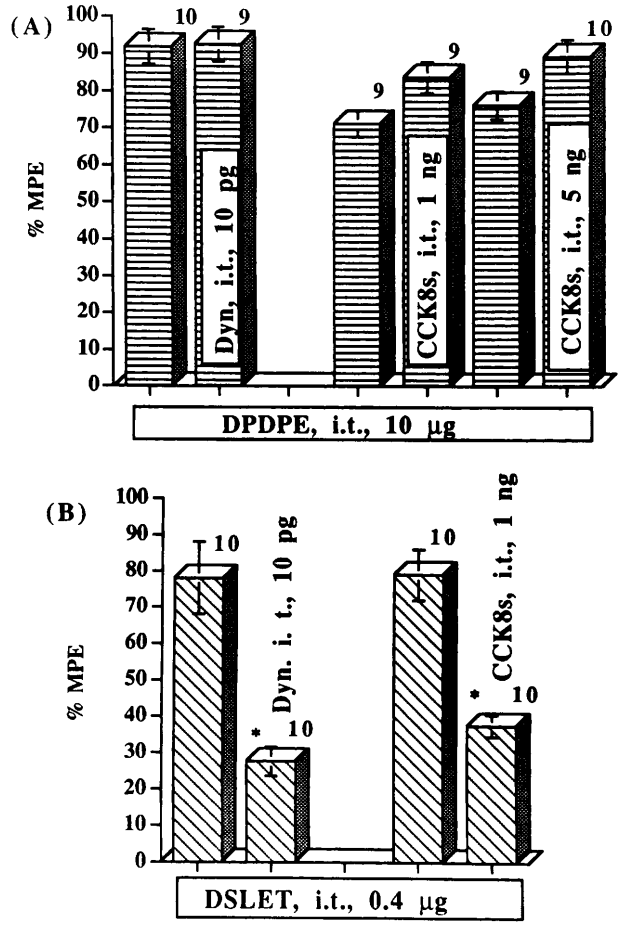

Figure 6. Evaluation of the ability of i.t. Dyn and CCK8s to inhibit the analgesic action of (panel A) DPDPE and (panel B) DSLET, δ_1 and δ_2 opioid receptor agonists, respectively. An asterisk (*) indicates a significant difference from the control ($P \leq 0.05$) by Students *t* test.

but not spinal Dyn (1, 2). Similarly, the antianalgesic action of i.c.v. neurotensin in mice involves CCK but not Dyn (Holmes BB *et al.*, unpublished data). Thus, the antianalgesic action of CCK does not involve Dyn.

Even though the antianalgesic actions of Dyn, pentobarbital (2), and neurotensin (3–5, Holmes BB *et al.*, unpublished data) involve release of spinal CCK as a common feature, it is not known if the same descending pathway from the brain to the spinal cord is involved. This matter has broader implications because there are other ways in which spinal CCK is released. An intriguing physiological mechanism is that associated with the conditioned safety response in rats. Rats conditioned to produce stress-induced analgesia (30) can also be conditioned to respond to a safety signal associated with turning the stress off (31). Response to the safety signal releases spinal CCK to terminate the stress-induced analgesia. Likewise, morphine-induced analgesia is terminated by the safety cue (31). These and other aspects of antianalgesia are discussed by Maier *et al.* (32). It appears that multiple factors can be involved in activating the antianalgesic action of spinal CCK, and it remains to be determined to what extent a common descending pathway is involved.

In the results in Figure 4, it appeared that PD135 158 shifted, though not significantly, the dose response curve of i.t. morphine to the left. Perhaps by using larger numbers of mice and optimized doses of PD135,158, a significant enhancement of morphine action might be found. Also, lorglumide treatment may have had a slight tendency to enhance the analgesic potency of low doses of morphine (Fig. 3). Enhancement of morphine action by treatment with CCK receptor antagonists in rats has been reported by many workers and appears to involve the CCK_B rather than CCK_A receptors (6, 7, 9, 33–39). PD135 158 is about 400 times more selective for the CCK_B than the CCK_A receptor (19), and lorglumide has about 140 times more selectivity for CCK_A than CCK_B receptors (18). It seems likely that CCK_B receptors are involved in the present study, but the data are insufficient to be conclusive. Lorglumide definitely antagonized Dyn (Fig. 3) as it did CCK8s action previously (2). Because lorglumide and PD135 158 did not have analgesic activity at the doses used (Table II), it is assumed that their antagonist action against CCK8s occurred through interaction at the CCK receptors. The enhancement of morphine analgesia is also assumed to occur by interaction of the antagonists at the CCK receptors. This enhancement is consistent with the evidence that morphine releases spinal CCK (35). As a further extension, the recent work by Friedrich and Gebhart (39) demonstrates that the enhancement of i.t. morphine-induced analgesia by proglumide is eliminated when the same experiment is performed in spinally transected rats. Spinal transection does not change the sensitivity to morphine itself. They suggest "that supraspinal sites are necessary for release of spinal CCK following i.t. morphine administration." Our interpretation is that CCK is not released by i.t. morphine after spinal transection, and some other factor controls spinal CCK release. Our work in mice demonstrates that i.t. morphine releases spinal Dyn (21). Spinal transection in mice, which does not affect i.t. morphine analgesia in the tail flick test, eliminates the antianalgesic action of i.t. Dyn (11). In Friedrich and Gebhart's study, i.t. proglumide would no longer enhance i.t. morphine analgesia because spinal transection would eliminate the brain component necessary for release of spinal CCK by i.t. morphine, that is, the influence of spinal Dyn mediated through the brain. Hopefully, completion of their work in the rat would support the present proposal that is based solely on work in mice.

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