Pentobarbital Antagonism of Morphine Analgesia Mediated by Spinal Cholecystokinin

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ABSTRACT

Pentobarbital administered intracerebroventricularly to mice has been shown previously to inhibit the analgesic action of morphine given intrathecally. The purpose of the present study was to examine the proposal that this antianalgesic action was mediated spinaly by cholecystokinin. First, intrathecal coadministration of cholecystokinin-8 sulfate (CCK8s) with morphine inhibited the analgesic action of morphine in the mouse tail-flick test. This rightward shift of the morphine dose-response curve was reversed by the intrathecal administration of either the CCKA receptor antagonist, lorglumide, or the CCKβ receptor antagonist, PD135,158. Second, lorglumide and PD135,158 given intrathecally also eliminated the antianalgesic effect of intracerebroventricularly administered pentobarbital against intrathecal morphine. Third, intrathecal pretreatment with CCK antiserum eliminated the effect of pentobarbital. Thus, the results indicated that pentobarbital antianalgesia was obtained through activation of a descending system to the spinal cord, where cholecystokinin inhibited the spinal analgesic action of morphine.

Barbiturates may enhance morphine-induced analgesia (Poutani et al., 1985) or, at certain dose ratios, antagonize the analgesic action of opioids (Jebeles et al., 1986; Poutani et al., 1985). It is thought that the antagonistic action is mediated through actions on the brain (Ding et al., 1990; Neal, 1965; Oasipov and Gebhart, 1984; Smith et al., 1992; Wang and Fujimoto, 1993), whereas enhancement is produced by an action on the spinal cord (Carlsson and Jurna, 1986; Jebeles et al., 1986; Stein et al., 1987; Wang and Fujimoto, 1993). Because pentobarbital given i.c.v. antagonizes the analgesic action of morphine given i.t., the antagonism appears to involve a descending modulatory mechanism (Wang and Fujimoto, 1993). Administration of midazolam i.c.v. also antagonizes i.t. morphine-induced analgesia. This latter antagonistic interaction is mediated by the antianalgesic action of dynorphin A(1–17) in the spinal cord (Rady and Fujimoto, 1993). Because pentobarbital given i.c.v. antagonizes the analgesic action of morphine given i.t., the antagonism appears to involve a descending modulatory mechanism (Wang and Fujimoto, 1993). The present study implicates a descending system which releases cholecystokinin in the spinal cord and accounts for the antianalgesic effect of pentobarbital.
CCK$_R$ receptor cloned from mouse brain shows high homology to that of the rat (Vitale et al., 1990; Wank et al., 1994).

Several different stimuli release spinal CCK. A physiologically important stimulus is associated with a safety signal. Stress-induced analgesia as well as morphine-induced analgesia is terminated when rats are given the cue for safety to which they were conditioned previously (Wiertelak et al., 1992, 1994). Stress-induced analgesia provoked by fear is terminated by a safety signal so that the rat is returned to its normally responsive state by activation of the CCK system, and release of CCK does not produce hyperalgesia (Wiertelak et al., 1992; Maier et al., 1992). The CCK system is not tonically active so that administration of CCK antagonists does not produce analgesia in the normal rat. Spinal CCK also is released by administration of morphine (Zhou et al., 1993). The action of morphine involves a balance between analgesic and antianalgesic systems (Maier et al., 1992). CCK release also is associated with the failure of acupuncture to induce analgesia in certain rats (Han et al., 1986).

Furthermore, increases and decreases in CCK levels in the spinal cord affect the analgesic action of morphine in chronic pain models (Stanfa et al., 1994).

The present investigation on the action of pentobarbital to inhibit morphine analgesia is based on the premise that pentobarbital releases CCK in the spinal cord. The approach took advantage of administering the pentobarbital i.c.v. to inhibit the antinociceptive action of morphine given at a separate site, i.t. (Wang and Fujimoto, 1993). This approach allowed assessment of the involvement of spinal CCK8 action through the use of CCK$_K$ and CCK$_B$ receptor antagonists given i.t. at a site downstream from that of pentobarbital. The initial experiments confirmed that i.t. administration of CCK8s inhibited the antinociceptive action of i.t. morphine in the mouse tail-flick test. The i.t. administration of CCK receptor antagonists then eliminated this inhibition. Similarly, i.c.v. pentobarbital inhibition of i.t. morphine antinociception was evaluated in the presence and absence of these CCK antagonists given i.t. Also, i.t. administration of an antiserum to CCK was shown to eliminate the antianalgesic action of pentobarbital.

**Methods**

**Animals and treatments.** Adult male CD-1 mice, weighing between 25 and 30 g, were obtained from Sasco Laboratories (Omaha, NE). Each animal was used only once. All studies involved drug solutions or vehicle solutions given i.t. in a volume of 5 µl as described by Hylden and Wilcox (1980). The i.t. injections were made 5 min before the tail-flick test. This time corresponded to peak time of action of the drug as used in previous studies (Fujimoto et al., 1990; Rady and Fujimoto, 1993) or determined as stated. The drugs and usual doses were as follows: morphine, 1 µg (1.32 nmol); CCK8s, 5 ng (4.38 pmol); lorglumide, 1 µg (2.08 nmol); and PD135,158, 100 ng (123 pmol). Exceptions to the time of administration and doses (as for the studies to determine duration of action and dose-response relationships) are stated in “Results.” The i.c.v. route was used to administer a 100-µg (402 nmol) dose of pentobarbital or saline in a volume of 4 µl by the method of Haley and McCormick (1957) under light halothane anesthesia. This time and dose for pentobarbital was published previously (Wang and Fujimoto, 1993). Unless stated otherwise, 10 mice were used in each group. The CCK8 and control antiserum were given i.t. 1 hr before the tail-flick test based on the experience with dynorphin antiserum (Fujimoto et al., 1990; Holmes and Fujimoto, 1993).

**Tail-flick test.** The radiant heat TFT was performed as described by D’Amour and Smith (1941) with a beam of high-intensity light focused on the dorsal surface of the tail. The response latency between the onset of the radiant heat stimulus and the movement of the tail out of the light beam, which automatically turned off the stimulus, was determined. The light intensity was set to provide a predrug response time of 2 to 4 sec. A cutoff time of 10 sec was used to prevent damage to the tail and was used as the maximum time. Two TFT trials were conducted before the administration of drugs, and the average was used as the predrug time. TFT response latencies in seconds were converted to percentage of maximum possible effect (%MPE) according to the formula (Dewey et al., 1970): %MPE = (postdrug time – predrug time) × 100/(10 – predrug time).

**Drugs.** The drugs were obtained from the following sources: sodium pentobarbital (Sigma Chemical Co., St. Louis, MO); morphine sulfate (Mallinckrodt Chemical Works, St. Louis, MO); and CCK8s (Peninsula Laboratories, Belmont, CA). The CCK$_K$ receptor antagonist, lorglumide sodium salt (Makevok et al., 1987; Kellstein et al., 1991) and CCK$_B$ receptor antagonist, PD135,158 N-methyl-glucamine (Hughes et al., 1990, 4-[2-[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[1,7,7-trimethylbicyclo [2,2,1]hept-2-yl-oxo]-carbonylaminopropyl]-1-phenylethyl]-amino]-1-phenylethylamine-4-oxo-[1S-(+)(+)]-bu- tanoate N-methyl-D-glucamine (bicyclo system 1S-endo) were obtained from Research Biochemicals International (Natick, MA). The CCK8 antiserum was obtained from Chemicon International Inc. (Temecula, CA). The control rabbit serum was that used previously (Fujimoto et al., 1990; Holmes and Fujimoto, 1993) and was produced by injecting male New Zealand rabbits with a combination of saline and complete Freund’s adjuvant. The doses used were for the form of the drugs as stated above. CCK8s was dissolved in a 0.01% (v/v) Triton X-100 solution in 0.9% (w/v) sodium chloride solution and other drugs were dissolved in a 0.9% (w/v) sodium chloride solution.

**Statistical analyses.** Group mean %MPE values were evaluated by analysis of variance followed by the Neuman-Keuls procedure for comparisons of multiple groups with each other, Dunnett’s test for comparisons of treatment groups with one control group and Student’s t test for comparisons between only two-group means (Steel and Torrie, 1960). Statistically significant differences were indicated by P ≤ .05. Slope and ED$_{50}$ values were determined and compared from a log dose vs. probit plot of the data by the method of Litchfield and Wilcoxon (1949) as described by Dewey et al. (1970).

**Results**

**Intrathecal CCK8s antagonism of i.t. morphine-induced antinociception.** The antinociceptive action of morphine (1 µg or 1.32 nmol) given i.t. 5 min before the TFT, was reduced by coadministration of the 1-, 10- and 100-ng doses of CCK8s (fig. 1A). At the 100-ng dose (87.5 pmol) of CCK8s, the antagonistic activity appears to have decreased somewhat, possibly because of antinociceptive actions of CCK8s (see “Discussion”). The antagonistic action for the 1-ng dose of i.t. CCK8s against i.t. morphine-induced antinociception was relatively short acting (fig. 1B). When CCK8s was given 20 min before the TFT, antagonism of morphine antinociception was still present as it was at the 5- and 15-min time points. However, at 30 min the antagonistic action was no longer significant. Doses of 1 and 10 ng of CCK8s did not produce any discernible antinociceptive or hyperalgesic response (table 1).

Dose-response curves for i.t. morphine were determined in the presence and absence of CCK8s (fig. 2). Morphine administered i.t. produced a dose-dependent antinociceptive response (open circles) with an ED$_{50}$ value (95% confidence interval) of 0.59 (0.36-0.96) µg (0.78 (0.47-1.27) nmol). Co-administration of CCK8s with the morphine resulted in a
parallel rightward shift (approximately 11-fold) of the i.t. morphine dose-response curve as demonstrated by the ED$_{50}$ value of 6.3 (3.2–12.5) μg [8.3 (4.22–16.47) nmol].

Elimination of the effect of CCK8s by lorglumide, a CCK$_A$ receptor antagonist. In figure 3A the antagonistic effect of CCK8s given along with morphine i.t. was reproduced in each of the three sets of experiments. The i.t. administration of lorglumide (a CCK$_A$ receptor antagonist) at doses of 0.25, 0.5 and 1 μg (2.08 nmol) reduced the antinociceptive antagonistic action of CCK8s against morphine in a dose-dependent manner (fig. 3A). The two larger doses eliminated the antagonistic effect of CCK8s. Treatment with i.t. lorglumide did not alter morphine antinociception in the absence of CCK8s and the combination of CCK8s and lorglumide did not affect the tail-flick response. The ability of the 1-μg dose of lorglumide to eliminate the CCK8s-induced antagonism of morphine antinociception was present when lorglumide was given up to 15 min before the TFT (fig. 3B). However, when the time of administration of lorglumide increased to 30 min, lorglumide no longer had an effect.

Returning to figure 2 for the dose-response curves for i.t. morphine, inclusion of lorglumide (1 μg) with the morphine and CCK8s i.t. (black squares) eliminated the antagonistic action of CCK8s. The ED$_{50}$ value for i.t. morphine in this combination was 0.13 (0.04–0.40) μg [0.17 (0.05–0.53) nmol], which indicates a leftward shift by approximately 48-fold compared with the curve showing the antagonistic action of CCK8s (black circles), ED$_{50}$ = 6.3 (3.2–12.5) μg [8.3 (4.22–16.47) nmol] and a 5-fold leftward shift compared with the control morphine dose-response curve (open circles), ED$_{50}$ = 0.59 (0.36–0.96) μg [0.78 (0.47–1.27) nmol]; P < .05.

Elimination of the effect of CCK8s by PD135,158, a CCK$_B$ receptor antagonist. Administration of PD135,158 (a CCK$_B$ receptor antagonist) also eliminated the antagonistic action of CCK8s on morphine antinociception (fig. 4). The protocol for this study was slightly different from those with lorglumide. In the lorglumide study (fig. 3A) consistent results were obtained with i.t. morphine, inclusion of lorglumide (1 μg) with the morphine and CCK8s i.t. (black squares) eliminated the antagonistic action of CCK8s. The ED$_{50}$ value for i.t. morphine in this combination was 0.13 (0.04–0.40) μg [0.17 (0.05–0.53) nmol], which indicates a leftward shift by approximately 48-fold compared with the curve showing the antagonistic action of CCK8s (black circles), ED$_{50}$ = 6.3 (3.2–12.5) μg [8.3 (4.22–16.47) nmol] and a 5-fold leftward shift compared with the control morphine dose-response curve (open circles), ED$_{50}$ = 0.59 (0.36–0.96) μg [0.78 (0.47–1.27) nmol]; P < .05.

Results presented in figure 2 indicated that PD135,158...
eliminated the antagonistic action of i.t. CCK8s as shown by dose-response curves for i.t. morphine. Compared with the antagonistic effect of i.t. CCK8s (black circles), ED_{50} = 6.3 (3.2–12.5) μg [8.3 (4.22–16.47) nmol], coadministration of PD135,158 i.t. produced a 9-fold shift of the curve to the left (black triangles), ED_{50} = 0.74 (0.37–1.5) μg [0.98 (0.49–1.98) nmol]. This latter ED_{50} value was not significantly different from that for the control morphine curve.

**Effect of lorglumide and PD135,158 indicate spinal CCK involvement in i.c.v. pentobarbital antagonism of i.t. morphine-induced antinociception.** As shown previously (29), pentobarbital given i.c.v. inhibited i.t. morphine-induced antinociception (fig. 5). As a new perspective, this antagonistic action was eliminated by i.t. administration of lorglumide (fig. 5A). The 0.5- and 1-μg (2.08 nmol) doses of lorglumide brought the morphine analgesia back to control levels. These doses were similar to those used to eliminate the antagonistic action of i.t. CCK8s against i.t. morphine (fig. 3). Also, administration of lorglumide and pentobarbital together without morphine did not produce an analgesic response. The duration of action of lorglumide was less than 30 min (fig. 5B) as it was earlier against i.t. CCK8s (fig. 3B).

The antagonistic effect of i.c.v. pentobarbital against i.t. morphine was also eliminated by i.t. administration of PD135,158 in a dose-dependent manner (fig. 6A). A 62.5-ng (77 pmol) dose of PD135,158 produced an intermediate effect, whereas the 250-ng (308 pmol) dose eliminated the morphine response. The duration of action of PD135,158 was similar to that shown earlier against CCK8s (fig. 4B).

Figure 7 presents the results in terms of dose-response curves for i.t. morphine. In the presence of i.c.v. pentobarbital, the ED_{50} for morphine was 4.81 (1.68–13.75) μg [6.34 (2.21–18.12) nmol]. This ED_{50} value was changed to 0.32 (0.14–0.69) μg [0.42 (0.18–0.91) nmol] by i.t. lorglumide and 0.81 (0.32–2.06) μg [1.07 (0.42–2.71) nmol] by i.t. PD135,158. The antagonistic effect of i.c.v. pentobarbital was eliminated, and the curves were shifted back to control values.
Elimination of the antagonistic effect of i.c.v. pentobarbital by i.t. administration of CCK8 antibody. An additional approach to implicating the release of CCK by i.c.v. pentobarbital was to determine whether administration of CCK8 antiserum would affect the system. In the study given in figure 8, CCK8 antiserum was administered 1 hr before the tail-flick test. At the 1:2000 dilution, a significant attenuation of the effect of i.c.v. pentobarbital-induced antagonism of morphine analgesia was obtained. Complete attenuation of the pentobarbital effect was obtained at the 1:1500 and 1:1000 dilution of the antiserum. These results provided further support for the primary proposal. Administration of saline, control antiserum and CCK8 antiserum (1:1000 dilution) alone did not significantly alter the tail-flick response as indicated by the %MPE ± S.E.M. values of 1.1 ± 2.1, −7.8 ± 2.4 and −1.0 ± 5.9, respectively.

Discussion

The results demonstrated that the analgesic action of i.t. morphine was antagonized by i.t. administration of CCK8s, which agrees with the work reported by others (see the introduction). Treatment with i.t. CCK8s produced a parallel shift to the right of the dose-response curve for morphine. This effect of CCK8s was eliminated by i.t. administration of the CCKA receptor antagonist, lorglumide, and the CCKB antagonist, PD135,158. The dose-response curve for morphine in the presence of lorglumide and CCK8s was shifted to the left of the morphine dose-response curve, an effect which might be related to the reports that CCK receptor antagonists enhance the action of morphine (Dourish et al., 1988, 1990a, b; Watkins et al., 1985a, b; Wiesenfeld-Hallin et al., 1990; Zhou et al., 1993). Morphine administration produces an increase in CCK release within the spinal cord (Benoliel et al., 1994; Zhou et al., 1993). Administration of the CCK antagonist inhibits the activity of this CCK leading to a more full expression of morphine antinociception. The enhancing effect of lorglumide on morphine analgesia was not investi-
gated any further because the phenomenon was not primary to the purpose of the present study. Also, no such enhancement of morphine analgesia was seen with Rady PD135,158 treatment. The reason for this difference between PD135,158 and lorglumide is unknown but may be the subject of a future investigation.

The premise that i.c.v. pentobarbital antagonized the analgesic action of i.t. morphine through the release of spinal CCK was investigated by the i.t. administration of lorglumide and PD135,158. Both treatments eliminated the antagonistic action of i.c.v. pentobarbital against i.t. morphine analgesia. The antagonism of the pentobarbital effect occurred in the same dose range and with similar duration of action as found for these antagonists against i.t. CCK8s. As in the CCK8s experiments, lorglumide produced a greater shift to the left than PD135,158 in antagonizing the effect of pentobarbital. Again no further experiments were performed to examine this difference. The fact that the 1-hr i.t. pretreatment with CCK8 antiserum eliminated the antagonistic effect of i.c.v. pentobarbital in a dose-dependent fashion (fig. 8) was also consistent with the expectation that an antibody to CCK8 should neutralize the effect of CCK released by the pentobarbital. Taken together, the evidence supports the proposal that pentobarbital antagonizes morphine analgesia by a release of spinal CCK8s. As envisioned, this pentobarbital action involves a descending modulation from the brain to the spinal cord. This directional feature rests on the combination of the sites of administration of the pentobarbital, i.e.; morphine, i.t.; and the CCK antagonists and CCK antiserum, i.t. In addition, the TFT relies on a spinal reflex that remains intact and suppressible by i.t. morphine after transection of the spinal cord in mice (Wang et al., 1994). Thus, the modulatory effect of i.c.v. pentobarbital on i.t. morphine is conceptualized as a descending influence from the brain to the spinal cord.

The mechanism through which pentobarbital acts on the brain to cause the release of spinal CCK may involve the benzodiazepine receptor in the brain. The benzodiazepine receptor antagonist, flumazenil, given i.c.v. inhibits the antianalgesic action of pentobarbital (Wang and Fujimoto, 1993). However, GABA receptors are not involved because bicuculline and picrotoxin have no effect. The antianalgesic action obtained through activation of brain benzodiazepine receptors is abolished by spinal transection (Rosland and Hole, 1990). Next, a connection is required between the descending action and the release of CCK in the spinal cord. If CCK present in the dorsal horn of the spinal cord seems to arise from neurons projecting downward from supraspinal sites like the periaqueductal gray area and the nuclear raphe magnus and from interneurons within the spinal cord (Skirboll et al., 1983; Zhang et al., 1993; Zouaoui et al., 1991; Jacquin et al., 1992; Mantyh and Hunt, 1984). Thus, pentobarbital given in the brain may activate the CCK-containing projection neurons or another descending neuronal system that acts on the spinal interneurons that contain CCK. Release of spinal CCK also is involved in the hyperalgesic action of small doses of neurotensin administered into the medullary nucleus raphe magnus of the rat (Urban et al., 1996) and the antianalgesic action of i.c.v. neurotensin in mice (B. B. Holmes, J. J. Rady, D. J. Smith and J. M. Fujimoto, et al., submitted). Even though there are multiple antianalgesic systems (Maier et al., 1992) some may impinge on common pathways. The involvement of CCK in both pentobarbital and neurotensin antianalgesia along with the fact that i.c.v. flumazenil inhibits the antianalgesic actions of both i.c.v.
pentobarbital (Wang and Fujimoto, 1993) and i.c.v. neurotensin in the mouse (B. B. Holmes, J. J. Rady and J. M. Fujimoto, unpublished data) suggests the possibility of a common antianalgesic pathway for the two agents. Other drugs that have antianalgesic action such as clonidine (Fujimoto et al., 1990; Rady et al., 1998, in press), midazolam (Rady and Fujimoto, 1993) and dynorphin A(1–17) (Rady and Fujimoto, 1993; Wang et al., 1994; Rady et al., 1998, in press) are being evaluated for spinal CCK release. A caveat in the mouse model is that CCK release is not measured chemically, and the evidence depends on functional measures of CCK effects.

The predominance of CCK \(_A\) receptors over CCK \(_B\) receptors in the central nervous system of rats is consistent with the ability of CCK \(_A\) receptor antagonists to inhibit the antianalgesic action of CCK (Stanfa et al., 1994; Weisenfeld-Hallin and Xu, 1996; Hill and Woodruff, 1990; Hill et al., 1990; Ghilardi et al., 1992). In the present study, both CCK \(_A\) and CCK \(_B\) receptor antagonists were effective in blocking CCK8s- and pentobarbital-induced antianalgesia. These results might arise from lack of sufficient selectivity of the antagonists for specific receptors. Lorglumide is approximately 140 times more selective for the CCK \(_A\) receptor than the CCK \(_B\) receptor, whereas PD135,158 is about 440 times more selective for CCK \(_B\) receptors than CCK \(_A\) receptors (Hughes et al., 1994; Makovec et al., 1987). Even though there are more selective antagonists (Hughes et al., 1990), lorglumide and PD135,158 were used because they are water soluble and commercially available. Another possible explanation for the present results is that both receptor types may be present in the mouse spinal cord. However, the issue requires further investigation.

The question of how CCK antagonizes morphine analgesia is covered in several recent reviews (Stanfa et al., 1994; Weisenfeld-Hallin and Xu, 1996). CCK receptors are found both presynaptically and postsynaptically to primary afferent fibers (Ghilardi et al., 1992) in a pattern similar to that of opioid receptors (Dickenson, 1991). Intrathecal morphine acts presynaptically to inhibit neurotransmitter release (Yaksh et al., 1995; Le Bars and Besson, 1981). CCK opposes the action of morphine by inhibiting intracellular Ca\(^{++}\) which increases transmitter release (Wang et al., 1992). Little is known about the postsynaptic action of CCK (Jeffinjia et al., 1981) in relation to its antianalgesic action.

In summary, as reported previously, spinal CCK modulates the analgesic response produced by i.t. morphine. The results demonstrate that spinal CCK receptors are also involved in i.c.v. pentobarbital antianalgesia of i.t. morphine analgesia. Thus, the present studies suggest that i.c.v. pentobarbital stimulates a descending neuronal system that either directly releases CCK or activates interneurons that release CCK within the spinal cord. It is this CCK that then inhibits the analgesic actions of morphine.


Smith DJ, Robertson F and Monroe PJ (1992) Antinociception from the administration of β-endorphin into the periaqueductal gray of rats is enhanced while that of morphine is inhibited by barbiturate anesthesia. *Neurosci Lett* 146:143–146.


