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 CCK_A receptor antagonist, lorglumide, or the CCK_B 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 CCK8s 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; Ossipov 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). However, dynorphin A(1–17) is not involved in the antagonistic action of pentobarbital (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.

Cholecystokinin present in the spinal cord as an octapeptide in the sulfated form, CCK8s (Hokfelt et al., 1994; Woodruff and Hughes, 1993), is well documented as having antioioid, antianalgesic actions (Baber et al., 1989). Faris et al. (1983) described the ability of CCK8s to antagonize morphine-induced analgesia in rats, an observation which has been extended by others (Magnunson et al., 1990; Stanfa et al., 1994; Wang et al., 1990; Wiesenfeld-Hallin and Duranti, 1987; Wiesenfeld-Hallin and Xu, 1996). Administration of CCK receptor antagonists eliminates the antagonistic action of CCK8s against morphine, enhances morphine analgesia, and inhibits the development of tolerance to morphine (Dourish et al., 1990a, b; Kellstein et al., 1991; Lavigne et al., 1992; Watkins et al., 1985a, b). The site of antioioid action of CCK8s appears to be in the dorsal horn where CCK and CCK receptors are localized at presynaptic nerve terminals on C-fibers (Stanfa et al., 1994; Wiesenfeld-Hallin and Xu, 1996, Kellstein et al., 1991; Mantyh and Hunt, 1984; Skirboll et al., 1983; Zouaoui et al., 1991) and where morphine also acts presynaptically on mu opioid receptors (Yaksh et al., 1995; Le Bars and Besson, 1981). In rodents, the CCK receptor found in the central nervous system is predominantly of the CCK_B subtype (Stanfa et al., 1994; Wiesenfeld-Hallin and Xu, 1996; Hill and Woodruff, 1990; Hill et al., 1990; Ghilardi et al., 1992), whereas the CCK_A subtype is found mainly in peripheral tissues (Woodruff and Hughes, 1993). However, the CCK_A subtype is the predominant form found in primate brains (Hill et al., 1990). Both CCK_A and CCK_B receptors have been cloned (Vitale et al., 1990; Wank et al., 1994). The

ABBREVIATIONS: i.c.v., intracerebroventricularly; i.t., intrathecal(ly); GABA, γ-aminobutyric acid; CCK8s, sulfated cholecystokinin octapeptide; ng, nanogram; %MPE, percentage of maximum possible effect; TFT, tail-flick test.

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CCK<sub>2</sub> 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 1992). The action of morphine involves a balance between analgesic and antinociceptive 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 CCK8s action through the use of CCK<sub>α</sub> and CCK<sub>β</sub> 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 injection of i.t. CCK8s was evaluated in the presence and absence of these CCK antagonists given i.t. Also, i.t. administration of an antisem to CCK was shown to eliminate the antinociceptive 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 TPT 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 TPT trials were conducted before the administration of drugs, and the average was used as the predrug time. TPT 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<sub>α</sub> receptor antagonist, lorglumide, sodium salt (Makovec et al., 1987; Kellettine et al., 1991) and CCK<sub>β</sub> receptor antagonist, PD135,158 N-methyl-D-glucamine salt (Hughes et al., 1990), 4-[[3-(3H-1-indol-3-yl)-2-methyl-1-oxo-2-[[1,7,7-trimethylbicyclo[2,2,1]hept-2-yl]oxy] carbonyl]amino]-1-phenylethylamino-4-oxo-[[S*](4S)[a]-butanato N-methyl-D-glucamine (bicyclic system 1S-endo) were obtained from Research Biochemicals International (Natick, MA). The CCK8s 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% (w/v) Triton X-100 solution in 0.9% (w/v) sodium chloride solution. All 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 two-group means 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<sub>50</sub> 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 TPT, 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 TPT, 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 administration produced a dose-dependent antinociceptive response (open circles) with an ED<sub>50</sub> 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) \( \mu g \) [8.3 (4.22–16.47) nmol], coadministration of lorglumide (1 \( \mu g \), 2.08 nmol) was given at various times before TFT in animals that were given the standard i.t. morphine and CCK8s treatments. * Indicates significant difference from all other groups within the given experiment; ** indicates significant difference from other groups not similarly marked within the given experiment (P < .05).

**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-\( \mu g \) (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) \( \mu g \) [6.34 (2.21–18.12) nmol]. This \( ED_{50} \) value was changed to 0.32 (0.14–0.69) \( \mu g \) [0.42 (0.18–0.91) nmol] by i.t. lorglumide and 0.81 (0.32–2.06) \( \mu g \) [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 determined in a protocol similar to that in figure 3. * Indicates significant difference from all other groups within the given experiment; ** indicates significant difference from other groups not similarly marked within the given experiment (P < .05).

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 CCK8 receptor antagonist, lorglumide, and the CCK8 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-
The premise that i.c.v. pentobarbital antagonized the antianalgesic 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 antianalgesic 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 antiseraum 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 antiseraum, 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. 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 nucleus 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.
the action of morphine by mobilizing intracellular Ca
acts presynaptically to inhibit neurotransmitter release
opioid receptors (Dickenson, 1991). Intrathecal morphine
et al. selective antagonists (Hughes Wiesenfeld-Hallin and Xu, 1996). CCK receptors are found
investigation. The mouse spinal cord. However, the issue requires further
commercially available. Another possible explanation for the
evidence depends on functional measures of CCK effects.
the evidence for CCKB receptors than CCKA receptors (Hughes et al., 1990; 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; Wiesenfeld-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 (Yakshe et al., 1995; Le Bars and Besslon, 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 antagonism 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.

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