Increased release of immunoreactive cholecystokinin octapeptide by morphine and potentiation of \( \mu \)-opioid analgesia by CCK\textsubscript{B} receptor antagonist L-365,260 in rat spinal cord

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This is the first report showing, in an in vivo study, that systemic morphine produced a marked (89\%, \( P < 0.01 \)) increase of the cholecystokinin octapeptide (CCK-8) immunoreactivity in the perfusate of the rat spinal cord, an effect completely reversed by naloxone. Since CCK-8 has been shown to possess potent anti-opioid activity at a spinal level, a blockade of the spinal cholecystokinin effect would be expected to potentiate opiate analgesia. With tail flick latency as a nociceptive index, it was found that intrathecal (i.t.) injection of a novel CCK\textsubscript{B} antagonist L-365,260 produced a marked potentiation of the analgesic effect induced by the \( \mu \)-opioid agonists morphine (4 mg/kg s.c.) or oxymorphone (32 ng i.t.). Similar effects were obtained with the CCK\textsubscript{A} antagonist devazepide at a dose 40–50 times higher than that of L-365,260. Both devazepide and L-365,260 showed a bell-shaped dose-response curve. The results confirm the notion that an increased release of CCK-8 may constitute a self-limiting process for opioid effects at the spinal level, and that it is the CCK\textsubscript{B} receptor which mediates the anti-opioid effect of CCK-8 in the rat spinal cord.

CCK receptor antagonists; CCK-8 immunoreactivity; CCK\textsubscript{B} receptors; Opiate analgesia; Naloxone

1. Introduction

Faris et al. (1983) were the first to show that cholecystokinin octapeptide (CCK-8) acts as a specific opiate antagonist in the rat spinal cord, as evidenced by the experimental finding that i.t. injection of 3.6 ng of CCK-8 antagonized opioid analgesia produced by footshock and morphine. This was confirmed by Han et al. (1985), who calculated that the dose of CCK-8 for 50\% reversal of the effect of morphine (5 mg/kg) analgesia in the rat was 1.7 ng and 3.1 ng by i.c.v. and i.t. routes of administration, respectively. Since CCK-8 has an abundant and wide-spread distribution in both brain and spinal cord, one would naturally anticipate that morphine or other endogenously released opioids would trigger the release of CCK-8 to counteract the opioid effect, and that removal of the effect of cholecystokinin would result in an augmentation of opioid analgesia and/or reversal of opiate tolerance. Experiments designed to test these hypotheses were not always convincing and successful due to a lack of adequate research tools. Thus, proglumide, a weak and non-specific CCK receptor antagonist, was shown to potentiate morphine analgesia in one report (Watkins et al., 1985) and to have no effect in another report (Lehman et al., 1989). Central administration of cholecystokinin antiserum was reported to potentiate analgesia induced by i.c.v. administered \( \beta \)-endorphin (Itoh et al., 1985) but not by systemically injected morphine (Ding et al., 1986), probably due to difficulties with the rapid diffusion of the antibody molecule from the cerebral ventricle into the brain tissue.

The development of the potent, specific, non-peptide CCK receptor antagonists, devazepide and L-365,031 for CCK\textsubscript{A} receptors (Chang and Lotti, 1986; Evens et al., 1986), and L-365,260 (Lotti and Chang, 1989) and PD134308 (Wiesenfeld-Hallin et al., 1990) for CCK\textsubscript{B} receptors, greatly facilitated research in this field. Dourish et al. (1990) have shown recently that morphine analgesia is significantly potentiated by L-365,260 and by the CCK\textsubscript{A} antagonist L-365,031 at a 20 times higher dose, suggesting that the CCK\textsubscript{B} receptor medi-
ates the anti-opioid effect. No information, however, is available concerning the site of action of these two specific antagonists for their anti-opioid activity.

Another basic question is whether morphine accelerates the release of CCK-8 from the CNS. Besides the in vitro studies performed on periaqueductal grey (Rattray and Delleroche, 1987), substantia nigra (Benoliel et al., 1990) and spinal cord (Benoliel et al., 1991), the most commonly cited in vivo study is the preliminary observation of Tang et al. (1984) that the addition of morphine (1 μM) to the fluid perfusing rat spinal subarachnoid space increases the cholecystokinin content of the perfusate. Since they did not present a statistical analysis in their preliminary report, it seemed worthwhile to re-evaluate this important issue in order to ascertain the extent and the time course of this reaction.

On the basis of the above mentioned information the present study was performed to clarify the following issues: (a) whether systemic injection of a conventional dose of morphine (5 mg/kg s.c.) would indeed accelerate the release of CCK-8 immunoreactivity from the rat spinal cord, and (b) whether the novel CCK_α receptor antagonist devazepide and the CCK_β receptor antagonist L-365,260 would potentiate opioid analgesia after i.t. injection and, if so, what is the dose ratio between the two antagonists.

2. Materials and methods

2.1. I.t. injection of drugs

Male Wistar rats weighing 200–250 g were anesthetized with chlorohydrate (0.4 g/kg i.p.). The i.t. cannula was implanted as described by Yaksh and Rudy (1976). A PE-10 polyethylene catheter was implanted through the cisterna magna down to the subarachnoid space for 7.5 cm to reach the upper border of the lumbar enlargement of the spinal cord. The outer part of the catheter was plugged and fixed to the skin after the wound was closed. Experiments with i.t. injection of drugs started about 20–26 h after the surgical operation. Drugs were injected via the catheter at a volume of 10 μl, followed by 10 μl of normal saline to flush out the cannula. Injection was completed within 30 s.

2.2. Perfusion of the subarachnoid space of the spinal cord

Rats were anesthetized with chlorohydrate, and a PE-10 catheter was inserted 7.5 cm down the subarachnoid space through the cisterna magna. Another polyethylene tubing (PE-50) was inserted 1 cm into the cisterna. Artificial cerebrospinal fluid containing captopril (1 μM) and bestatin (1 μM) was perfused at a rate of 1.0 ml/30 min with a push-pull pump.

2.3. Nociceptive test

The rat was restrained in a plastic holder with its hind legs protruding. The nociceptive threshold was measured by the latency of the tail flick response, elicited by radiant heat applied to the lower 1/3 of the tail. The mean tail flick latency of three measurements taken at 5-min intervals at the start of the experiment was taken as the basal threshold. The tail flick latency taken at 10-min intervals after drug administration is expressed as the percentage change from basal tail flick latency, with a cutoff limit of +150% to avoid unnecessary skin damage.

2.4. Radioimmunoassay of CCK-8

Cholecystokinin was extracted as reported by Marley and Rehfeld (1984). Aliquots of spinal perfusate (1.0 ml) were collected in polyethylene tubes in an ice-water bath and then heated for 10 min in a water bath at 95°C. After cooling, the aliquots were centrifuged at 10,000 × g for 15 min. Supernatants were kept at −20°C and dried in a vacuum dryer. The residue was taken up in 0.2 ml H_2O for the radioimmunoassay of CCK-8. The cholecystokinin antiserum, which was kindly provided by Dr. J.S. Hong (NIEHS, USA), showed no detectable cross reactivity with [Met^5]enkephalin, β-endorphin, gastrin, CCK-7, CCK-4, or unsulfated CCK-8. The radioimmunoassay procedure for cholecystokinin in the present study was similar to that suggested by Amersham Inc. On the basis of 10 consecutive assays, the sensitivity of the assay was 0.4 fmol/ml, with 4.6% intra-assay and 9.7% inter-assay variations.

2.5. Drugs

^{125}I-Bolting Hunter-CCK-8 was purchased from Amersham Inc. (UK). CCK-8 was a gift from Squibb and Sons, Inc. Morphine HCl was purchased from Qinhai Drug Company (China). Naloxone HCl was from Sigma (USA). Ohmefentanyl HCl (N-[1-(β-hydroxy-β-phenetyl)-3-methyl-4-piperidyl]-N-phenylproponamide) was obtained from the Shanghai Institute of Materia Medica. Devazepide (3S(-)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1-H-1,4-benzodiazepin-3-yl)-H-indole-2-carboxamide) and L-365,260 (3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)N'(3-methyl-phenyl)urea) were donated by Dr. R.F. Freidinger of the Merck, Sharp and Dohme Research Laboratories (USA). Devazepide and L-365,260 were first dissolved in dimethylsulfoxide (DMSO) and then diluted with propylene glycol to a
final ratio of 20% DMSO : 80% propylene glycol (v/v). Morphine HCl, naloxone HCl and ohmefentanyl were dissolved in normal saline.

2.6. Statistical analysis

Data were analyzed using a multifactorial analysis of variance (ANOVA). Where ANOVA revealed a significant effect, between group comparisons were made using Duncan’s test. Student’s t-test was used for group comparisons where appropriate. A probability level of 0.05 was regarded as significant.

3. Results

3.1. Increase in CCK-8 immunoreactivity in spinal perfusates elicited by systemic morphine and the prevention of this effect by i.t. naloxone

Three groups of eight rats were used in the spinal perfusion experiments. Each group was perfused for three 30-min perfusion periods at a rate of 1 ml/30 min. At the end of the first perfusion period, an i.t. injection of normal saline (10 μl) or naloxone (20 μg) was given, followed 10 min later by a s.c. injection of normal saline (0.5 ml/rat) or morphine (5 mg/kg). Spinal perfusion was started again immediately after the s.c. injection, which lasted for 60 min (2 × 30 min). Perfusate was collected as 30-min fractions (1 ml).

Radioimmunoassay revealed that in the control group (fig. 1, left panel) the content of CCK-8 immunoreactivity remained rather stable within the range of 14.4 to 15.2 fmol/ml in the three perfusion periods. Administration of morphine resulted in an 89% increase in CCK-8 immunoreactivity in the first post-morphine period (P < 0.01 compared with the pre-morphine period) and a 32% increase in the second post-morphine period (fig. 1, middle panel). This effect of morphine was totally reversed by naloxone (20 μg i.t.) administered 10 min prior to morphine (fig. 1, right panel).

3.2. The effect of devazepide and L-365,260 on nociceptive threshold

To assess the effect of the CCK receptor blockers on tail flick latency, successive doses of devazepide (10, 25, 50, 100, 200 and 400 ng) or L-365,260 (0.625, 1.25, 2.5, 5.0, 10 and 20 ng) in 10 μl volumes were administered i.t. at 20-min intervals. As can be seen from fig. 2A and B, no significant changes in tail flick latency were observed over a period of 140 min.

3.3. Potentiation of morphine analgesia by devazepide or L-365,260 administered i.t.

Two groups of nine rats were injected i.t. with 100 ng of devazepide or 10 μl of vehicle, followed 15 min later by a s.c. injection of 4 mg/kg morphine. The results are shown in fig. 3A. The antinociceptive effect of morphine was significantly higher in the devazepide group than in the control group (P < 0.01, ANOVA for the 60-min observation period). A similar experiment was performed with normal saline instead of morphine. As can be seen from fig. 3A, no significant increase in tail flick latency was found in the two groups (ANOVA, P > 0.05), indicating that devazepide alone was not analgesic.

The above experiments were repeated with L-365,260 (2.5 ng) instead of devazepide (100 ng), and the results are shown in fig. 3B. L-365,260 produced a marked potentiation of morphine analgesia (P < 0.01, ANOVA for the 60-min observation period). Injection of L-365,260 alone did not produce significant changes in tail flick latency.

The dose-response curves for the effect of the two CCK receptor blockers on morphine analgesia are shown in fig. 4, where changes in tail flick latency were recorded 30 min after morphine injection (at the peak effect). From the two bell-shaped dose-response curves, optimal doses of devazepide and L-365,260 for potentiation of morphine analgesia were calculated to be 100 and 2.5 ng, respectively, showing that the CCK_β blocker was 40 times more effective than the CCK_A blocker on a weight basis and 37 times more effective on a molar basis.
Fig. 2. Changes in tail flick latency after successive i.t. injections of devazepide (A) and L-365,260 (B). Mean tail flick latency ± S.E.M. is plotted against time in min. Devazepide or L-365,260 was injected in 10 μl at 20-min intervals. The doses are shown under the arrows.

Fig. 3. Time course of potentiation of morphine analgesia by devazepide (A) and L-365,260 (B). Percent change in tail flick latency is plotted against time in min. Morphine or normal saline (NS) was injected s.c. at time 0, and devazepide, L-365,260 or vehicle was injected i.t. 15 min prior to morphine or NS, as indicated by the arrows. For symbols in panel A: (●) devazepide + morphine (9), (▲) devazepide + NS (9), (○) vehicle + morphine (9), (△) vehicle + NS (7). For symbols in panel B: (●) L-365,260 + morphine (9), (▲) L-365,260 + NS (9), (○) vehicle + morphine (9), (△) vehicle + NS (6).

Fig. 4. Dose-response curves for the potentiation of morphine analgesia by the CCK<sub>A</sub> antagonist devazepide (A) and the CCK<sub>B</sub> antagonist L-365,260 (B) in the rat tail flick test. Shown are data recorded 30 min after the injection of morphine (4 mg/kg s.c.). Devazepide, L-365,260 or vehicle was injected i.t. 15 min prior to morphine. Between group comparisons were made with an ANOVA, followed by Duncan test. * P < 0.05 as compared to vehicle/morphine (V) treatment. V/S: vehicle/normal saline control.
3.4. Potentiation of ohmefentanyl-induced analgesia by devazepide and L-365,260 administered i.t.

Unlike morphine, which is an opioid agonist with a relative preference for μ-opioid receptors, ohmefentanyl is one of most selective μ-opioid agonist available today (Xu et al., 1985; Goldstein and Naidu 1990). As the site of interaction between opioids and cholecystokinin would be made clearer if both agonists were administered at the spinal level, we decided to see whether the analgesia induced by i.t. ohmefentanyl would be potentiated by i.t. injected CCK receptor antagonist.

Two groups of nine rats were given an i.t. injection of 66 ng of devazepide or 10 μl of vehicle, followed 10 min later by an i.t. injection of 32 ng of ohmefentanyl. As shown in fig. 5A, ohmefentanyl-induced analgesia was significantly potentiated by devazepide (P < 0.05, ANOVA for the 60-min observation period). A similar experiment was performed with normal saline instead of ohmefentanyl. No significant changes in tail flick latency were observed in the two groups, indicating that devazepide alone was not analgesic.

The above experiments were repeated with L-365,260 (1.25 ng) instead of devazepide (66 ng). The results are shown in fig. 5B. Analgesia induced by ohmefentanyl was markedly potentiated by L-365,260, both administered via the i.t. route (P < 0.01, ANOVA for the whole course of observation period), and L-365,260 per se was not analgesic.

Dose-response curves were made for the effect of devazepide (fig. 6A) and L-365,260 (fig. 6b) on ohmefentanyl-induced analgesia. The ordinates show the peak analgesic effect to occur at 30 min for devazepide and 40 min for L-365,260. The optimal doses for potentiation of ohmefentanyl analgesia were 66 ng for de-
vazepide and 1.25 ng for L-365,260, as determined from the two bell-shaped dose-response curves. Thus the CCK\textsubscript{B} receptor antagonist was 53 times more effective than the CCK\textsubscript{A} receptor antagonist on a weight basis and 49 times more effective on a molar basis.

4. Discussion

In our hands, the analgesic effect induced by a moderate dose (5 mg/kg s.c.) of morphine, as tested by the tail flick latency, usually peaks at 30 min and subsides at 60 min. Measurement of CCK-8 immunoreactivity in spinal perfusate revealed that the time course of the cholecystokinin reaction almost paralleled the opiate effect: a 89% increase within 30 min and then a drop to a 32% increase in the second 30-min period. Thus the increased release of CCK-8 can be regarded as a fast negative feedback control for the morphine effect. In another study we measured CCK-8 immunoreactivity in the cisternal CSF of rats that had received chronic morphine treatment for 6 days. There was a 70% increase (P < 0.01) on the first day, a 39% increase (P < 0.05) on the third day, and a return to the control level on the sixth day, when tolerance was fully developed (Pu, Zhou and Han, to be published). Therefore the cholecystokinin reaction seems to be phasic rather than static in nature. That the effect of morphine was mediated by opiate receptors in the spinal cord was shown by the observation that it was totally abolished by i.t. naloxone.

The notion that the spinal cord is a strategic site for cholecystokinin/opiate interactions is supported by the finding that (a) the analgesic effect of systemic morphine was markedly potentiated by cholecystokinin blockers administered intrathecally, and (b) the interaction between opiate agonist and cholecystokinin receptor antagonist took place when both drugs were administered into the spinal subarachnoid space.

According to Dourish et al. (1990), the optimal dose for L-365,260 to potentiate morphine analgesia is 0.2 mg/kg i.p. In a 250-g rat it would be equivalent to 50 \(\mu\)g per rat. Assuming an even distribution of the drug in the whole body, the optimal dose for i.t. injection of L-365,260 to potentiate morphine analgesia would be approximately 100 ng which was 40 times higher than the optimal dose found in the present study (2.5 ng i.t.). A blood-brain barrier therefore seems to exist for this non-peptide molecule.

The finding that the CCK\textsubscript{B} antagonist was 40–50 times more effective than the CCK\textsubscript{A} receptor antagonist is in agreement with that reported by Dourish et al. (1990), who found a 20-fold difference between the CCK\textsubscript{B} receptor antagonist L-365,260 (0.2 mg/kg i.p.) and the CCK\textsubscript{A} antagonist L-365,031 (4 mg/kg i.p.). It is also in line with the finding that CCK\textsubscript{B} receptors are present in the rat spinal cord (Dourish and Hill, 1987).

We have reported recently that the effect of CCK-8 on opioid binding depends on the type of opiate receptor studied. Thus CCK-8 decreased the \(K_{d}\) of \(\mu\)-opioid sites and increased the \(K_{d}\) of \(\kappa\)-opioid sites, without affecting \(\delta\)-opioid sites (Wang and Han, 1990). Since morphine is an opioid agonist that recognizes both \(\mu\)- and \(\delta\)-opioid sites, it was of interest to compare the effect of cholecystokinin on the response to morphine with that of a pure \(\mu\)-opioid agonist. The novel fentanyl derivative ohmefentanyl has been characterized as one of the most selective \(\mu\)-opioid agonists available today (Goldstein and Naidu, 1990). The results shown in fig. 3–6 indicate that the profile of the cholecystokinin antagonism of ohmefentanyl-induced analgesia was almost identical to that of morphine, with an optimal L-365,260 dose of 1.25 ng as compared to 2.5 ng in the case of morphine, and a devazepide/L-365,260 ratio of 49 as compared to 37 in the case of morphine. From a theoretical point of view, it would be interesting to look at the effect of cholecystokinin on the analgesia produced by dynorphin, an endogenous \(\kappa\)-opioid agonist, because it has been shown that CCK-8 exerts a modulating effect on the affinity of \(\kappa\)-opioid sites but causes a down-regulation of \(\mu\)-opioid sites (Wang and Han, 1990). We found recently that L-365,260 was much more effective in potentiating the analgesic effect elicited by high-frequency (100 Hz) electroacupuncture, which is known to be mediated by the \(\kappa\)-opioid agonist dynorphin in the spinal cord, than it was in potentiating the analgesic effect elicited by low-frequency (2 Hz) electroacupuncture, which is mediated by the \(\delta/\mu\) agonist [Met\textsuperscript{\text{5}}]enkephalin (Shen, Zhou and Han, to be published).

Since the analgesic effect induced by ohmefentanyl, a pure \(\mu\)-opioid agonist, was potentiated by a CCK receptor antagonist, it could be reasoned that \(\mu\)-opioid receptor activation causes cholecystokinin release. However, in vitro (Benoliel et al., 1991) and in vivo (Rodriguez and Sacristan, 1989) studies have shown that the \(\mu\)-opioid agonist DAGOL ([D-Ala\textsuperscript{2}, MePhe\textsuperscript{4}, Gly\textsuperscript{\text{5}}]enkephalin) suppresses CCK-8 release in the spinal cord, whereas the \(\delta\)-opioid agonist DTLET ([D-Thr\textsuperscript{2},Leu\textsuperscript{\text{5}}]enkephalin-Thr\textsuperscript{\text{8}}) activates cholecystokinin release from rat spinal cord slices (Benoliel et al., 1991). These authors suggested that the effect of morphine to increase cholecystokinin release is mediated mainly through \(\delta\)-opioid receptors. Consequently, interpretation of the potentiation by CCK receptor antagonists of the analgesic effect of morphine is far from conclusive.

The old puzzle of the bell-shaped dose-response curve for CCK receptor blockers on opiate analgesia was again prominent in the present study, even when both drugs (opiate agonist and CCK receptor antago-
nist) were administered i.t. No adequate explanation can be provided at the present time. In this context, it is interesting to mention that Wiesenfeld-Hallin et al. (1990) reported that morphine analgesia (hot-plate test in rats) was potentiated by PD134308, a selective antagonist of CCK<sub>B</sub> receptor developed by Hughes and his group (1990). The potentiation increased steadily over the dose range of 0.1–1.0 mg/kg. However, it is important to note that PD134308 per se became analgesic at 1.0 mg/kg or higher, and the suppressive effect of PD134308 (1 mg/kg) on the rat spinal flexor reflex was shown to be naloxone reversible. It is obvious that a bell-shaped curve would not appear if the PD compound caused opioid release at higher doses, although the later possibility needs to be verified experimentally.

In conclusion, the spinal cord is an important area for cholecystokinin/opioid interactions, and the CCK receptors involved are of the CCK<sub>B</sub> type. Under physiological conditions, the amount of CCK-8 released is very low, which may explain the fact that CCK receptor blockers did not significantly influence the basal nociceptive threshold. Administration of an opioid may accelerate the release of CCK-8 in the spinal cord, thereby forming a negative feedback control on the opioid effect. Removal of this negative control resulted in an augmentation of the opioid effect. It should be pointed out that while the tonicity of both opioid and anti-opioid activities is low under normal physiological conditions, it may shift to a high tonicity status in situations such as chronic pain (Przewlocki et al., 1986; Höltt et al., 1987). Chronic pain might activate the cholecystokinin system via an increased release of endogenous opioids (Przewlocki et al., 1986), and CCK blockers given alone could be analgesic.

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