Circuitry Underlying Antiopioid Actions of Cholecystokinin Within the Rostral Ventromedial Medulla

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INTRODUCTION

It is now well established that the analgesic actions of opioids can be modified by endogenous “antiopioid” peptides, and the role of cholecystokinin (CCK) as a physiological opioid antagonist has received particular attention. Administration of CCK agonists can diminish opioid analgesia (Faris et al. 1982; Li and Han 1989), whereas CCK antagonists can slow or prevent the development of opioid tolerance in some paradigms (Chapman et al. 1983; Itoh et al. 1982; Li and Han 1989), whereas CCK acting within the brain to modify opioid analgesia. The aim of the present study was to characterize the actions of CCK in a brain region in which the circuitry mediating the analgesic actions of opioids is relatively well understood, the rostral ventromedial medulla (RVM). Single-cell recording was combined with local infusion of CCK in the RVM and systemic administration of morphine in lightly anesthetized rats. The tail-flick reflex was used as a behavioral index of nociceptive responsiveness. Two classes of RVM neurons with distinct responses to opioids have been identified. OFF cells are activated, indirectly, by morphine and μ-opioid agonists, and there is strong evidence that this activation is crucial to opioid antinociception. ON cells, thought to facilitate nociception, are directly inhibited by opioids. Cells of a third class, NEUTRAL cells, do not respond to opioids, and whether they have any role in nociceptive modulation is unknown. CCK microinjected into the RVM by itself had no effect on tail flick latency or the firing of any cell class but significantly attenuated opioid activation of OFF cells and inhibition of the tail flick. Opioid suppression of ON-cell firing was not significantly altered by CCK. Thus CCK acting within the RVM attenuates the analgesic effect of systemically administered morphine by preventing activation of the putative pain inhibiting output neurons of the RVM, the OFF cells. CCK thus differs from another antiopioid peptide, orphanin FQ/nociceptin, which interferes with opioid analgesia by potently suppressing all OFF-cell firing.

The focus of much of the recent work concerned with CCK-opioid interactions has been at the level of the spinal cord (Stanfa et al. 1994; Wiesenfeld-Hallin et al. 1999), and although CCK has also been shown in behavioral studies to act within the brain to inhibit opioid analgesia (Kovelowski et al. 2000; Li and Han 1989; Mitchell et al. 1998; Noble et al. 1993; Pu et al. 1994; Vanderah et al. 1996; Watkins et al. 1985b), the neural circuitry through which this occurs has not been identified. The goal of the present experiments was to determine how CCK influences opioid effects on the circuitry within the rostral ventromedial medulla (RVM), a region with a well-documented role in opioid analgesia (Basbaum and Fields 1984; Fields et al. 1991; Heinricher and Morgan 1999).

Three physiologically distinct populations of neurons have been identified in the RVM. Only one group of RVM neurons, OFF cells, is activated by μ-opioid agonists (Fields et al. 1983b; Heinricher et al. 1994). We have shown that this activation is indirect (Heinricher et al. 1992) and sufficient to produce behaviorally measurable antinociception (Heinricher and Tortorici 1994; Heinricher et al. 1994). Cells of a second class, ON cells, display a sudden increase in activity beginning just before the occurrence of nocifensive reflexes, and likely exert a permissive or even facilitating effect on nociception. ON-cell firing is directly inhibited by μ-opioid agonists (Bederson et al. 1990; Fields 1992; Heinricher et al. 1992; Pan et al. 2000). NEUTRAL cells show no change in activity associated with nociceptive responses and do not respond to opioids given by any route (Barbaro et al. 1986; Gao et al. 1998). Their role, if any, in nociceptive modulation remains unclear. The advantages to using the RVM for this analysis of CCK-opioid interactions are thus that the responses of RVM neurons to opioid...
administration are known and that how each cell class contributes to opioid analgesia is relatively well understood.

CCK has been shown to have excitatory effects in a number of brain regions, including the hippocampus, nucleus tractus solitarius, dorsal horn, and periaqueductal gray, although inhibitory effects are sometimes seen (Albrecht et al. 1994; Boden and Woodruff 1994; Jeffinijia et al. 1981; Liu et al. 1994; Miller et al. 1997). Thus a reasonable hypothesis would be that CCK acting in the RVM could disrupt morphine antinociception by activating ON cells, blocking opioid inhibition of these neurons. We tested this possibility by examining the effects of locally applied CCK on the activity of RVM neurons and their responses to systemically administered morphine. We found that, contrary to expectation, CCK attenuated the analgesic actions of systemically administered morphine by interfering with opioid activation of OFF cells.

METHODS

Animals and surgical preparation

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Oregon Health Sciences University. Male Sprague-Dawley rats (250–300 g, B and K, Hayward, CA) were anesthetized with pentobarbital (60 mg/kg ip), and catheters were inserted into the external jugular veins for administration of anesthetic, morphine, and naloxone. The rat was placed in a stereotaxic apparatus, a hole was drilled in the skull over the cerebellum, and the dura was removed to allow placement of an electrode/microinjection cannula assembly in the medulla. Body temperature was maintained at approximately 37°C by a circulating-water pad.

Following surgery, the anesthetic level was allowed to lighten until the tail-flick response (TF), a spinal nociceptive reflex, could be elicited by application of noxious heat using a feedback-controlled projector lamp focused on the blackened ventral surface of the tail. The animals were then maintained in a lightly anesthetized state with a continuous infusion of methohexital (15–30 mg·kg⁻¹·h⁻¹ iv) as previously described (Barbaro et al. 1989).

nociceptive testing

TF latency was used as a measure of nociceptive responsiveness. The heat was applied to spots 2, 3, or 4 cm from the tip of the tail in succession. Each trial consisted of a linear increase in temperature at approximately 1.8°C/s from a holding temperature of 34°C until the TF occurred or to a maximum of 52°C at 10.6 s. TF trials were carried out at 5-min intervals throughout the experiment.

Recording and drug administration

A gold- and platinum-plated stainless steel recording microelectrode (Frederick Haer, Brunswick, ME) was glued parallel to a single-barrel glass infusion pipette with an outer diameter of 75–80 μm in such a way that the tips were separated laterally by 100–300 μm. This separation allowed us to maintain well-isolated recordings of single neurons during and after infusions of 200 nl of saline vehicle or drug solution in the RVM in a reasonable number of experiments. The assembly was oriented in the electrode carrier so that the assembly straddled the midline, with both electrode and infusion pipette within the RVM. The infusion pipette was attached to a 1 μl Hamilton syringe with a length of PE-50 tubing for drug infusion.

RVM neurons were classified as previously described (Fields et al. 1983a). Spike waveforms were monitored and stored for off-line analysis (Datave System, Thornton, CO) to ensure that the unit under study was unambiguously discriminated throughout the experiment and to verify that the solutions applied did not have local anesthetic effects. Spike times were stored with a temporal resolution of 0.1 ms. OFF cells were characterized by an abrupt pause in ongoing activity beginning just prior to the occurrence of the TF. On cells were identified by a sudden burst of activity beginning just prior to the occurrence of the TF. Cells of a third class, neutral cells, were identified by no change in activity associated with the tail flick or with noxious or innocuous somatic stimulation.

Protocol and data analysis

One set of experiments examined the effect of systemic administration of CCK on the ability of systemically administered morphine to alter TF latency and RVM neuronal firing. Following three baseline TF trials, CCK-8S (Tocris Cookson, CCK), the predominant CCK peptide in the CNS (Rehfeld et al. 1985), was given intravenously (4 μg/kg). This was followed 10 min (2 TF trials) later by systemic administration of morphine sulfate (MS) (0.375–1.5 mg/kg iv). TF latency and cell activity were then monitored for an additional 40 min.

In a second group of animals, we determined the effect of CCK microinjection into the RVM on TF latency and the ongoing and TF-related discharges of RVM neurons. Following three baseline TF trials, CCK (10 ng) was infused into the RVM. This dose was chosen because pilot studies had shown that microinjection of 10 ng CCK into the RVM had an effect on morphine antinociception that was similar to that of 4 μg given intravenously. All RVM injections were made in a volume of 200 nl over a period of approximately 4 min. TF latency and cell activity were then monitored for a period of 45 min.

A third set of experiments used a similar paradigm to investigate the effect of CCK microinjection into the RVM on the actions of systemically administered morphine. Following three baseline TF trials, CCK (10 ng) or saline vehicle was infused into the RVM. RVM infusions were followed 10 min (2 TF trials) later by systemic administration of morphine sulfate (0.375–1.5 mg/kg iv). TF latency and cell activity were then monitored for an additional 40 min.

Only one protocol was performed in each of the 249 animals used in these experiments. Data are presented only from experiments in which the neuron was successfully held throughout the entire testing period.

TF latencies and cell parameters obtained in the baseline period were compared with the average of the post-CCK or the 15- through 30-min postmorphine time points. Inhibition of the TF was quantified as percent maximum possible effect, i.e., %MPE = 100*(postdrug latency – baseline latency)/(10.6 – baseline latency). Three cell parameters were analyzed. 1) Ongoing activity. Because OFF and ON cells often show irregular alternations between periods of silence and activity, cell activity integrated over the 30 s prior to each TF trial was used as an overall index of ongoing firing. 2) The OFF-cell pause. The TF-related OFF-cell pause in firing was analyzed for those tail flick trials on which the OFF cell was active immediately prior to heat onset. TF-related inhibition of firing was calculated by expressing the firing rate measured in the 2-s period beginning 1 s before the TF (or, if no TF occurred following morphine administration, the mean TF latency in baseline) as a percentage of that in the 5-s epoch immediately prior to heat onset. A value of 100% would thus indicate no slowing associated with tail heat/flick, a value of 0% complete inhibition for at least 2 s. These intervals were chosen based on the previously described spontaneous firing pattern of RVM neurons and duration of the OFF-cell pause (Barbaro et al. 1989). 3) ON-cell TF-related burst. Total spike count in the 3-s period beginning 1 s before the TF was recorded for all TF trials.

Data are presented as means ± SE. Wilcoxon’s signed ranks test and Mann-Whitney U (for TF latencies), ANOVA and Student’s t-test (for cell parameters) were used for statistical analysis of results; P < 0.05 was considered significant.
Histology

At the conclusion of the experiment, recording/infusion sites were marked with an electrolytic lesion. Animals were killed with an overdose of methohexital and perfused intracardially with physiological saline followed by 10% formalin. Recording/infusion sites were histologically verified and plotted on standardized sections (Paxinos and Watson 1997). The distribution of sites was within the RVM, as previously defined, comprising the nucleus raphe magnus as well as the laterally adjacent reticular formation at the level of the facial nucleus, including the nucleus reticularis gigantocellularis pars alpha and nucleus paragigantocellularis lateralis.

RESULTS

Initial experiments were designed to identify a dose and protocol for CCK attenuation of morphine antinociception. TF latencies of the RVM-saline-injected, intravenous-saline, and no-treatment control groups did not differ (1-way ANOVA), and these three groups were combined for comparison with animals that received CCK. There was no difference in baseline TF latencies among the control or CCK groups (mean ± SE: 4.73 ± 0.08 s). As shown in Fig. 1, morphine given intravenously produced a dose-related increase in TF latency in the control animals, and this was significantly attenuated by administration of 4 µg/kg CCK intravenously 10 min prior to the morphine infusion. CCK given after morphine administration did not reverse morphine antinociception (data not shown).

We then examined the effects of CCK microinjected into the RVM on TF latency and RVM neuronal activity. As with systemic administration, microinjection of CCK into the RVM significantly attenuated the antinociceptive effect of systemically administered morphine (Fig. 1). CCK (10 ng) microinjected dorsal to the RVM did not alter the antinociceptive effect of morphine (%MPE for 0.75 mg/kg MS: 86.0 ± 12.6%, not significantly different from that of control animals, n = 4). Moreover, as shown in Fig. 2, CCK microinjection by itself had no effect on TF latency or the ongoing activity of ON, OFF, or NEUTRAL cells. In addition, the TF-related ON-cell burst was not altered (113 ± 14% of pre-CCK baseline) nor was the

**FIG. 1.** Cholecystokinin (CCK) given systemically or applied locally within the rostral ventromedial medulla (RVM) attenuates the antinociceptive effect of systemically administered morphine. Systemically administered morphine produced a potent, dose-related increase in tail-flick response (TF) latency (measured as percent of maximum possible effect, %MPE) in control animals. The antinociception was significantly attenuated by CCK given systemically (4 µg/kg iv) or microinjected into the RVM (10 ng/200 nl). (**$P < 0.01$ compared with baseline, $## P < 0.01$ compared to control group, n = 4–17 animals per CCK-treated group, 18–29 animals per control group.)

**FIG. 2.** Local application of CCK within the RVM by itself has no effect on TF latency or the ongoing activity of OFF, ON, or neutral cells. TF latencies and cell firing in the 45-min period following microinjection of CCK into the RVM are expressed as a percentage of that during the 15-min period baseline period. ($P > 0.05$ compared with baseline in each case, 15 animals, 6 ON cells, 6 OFF cells, and 4 neutral cells.)

TF-related off-cell pause (TF-related inhibition was 116 ± 11% of that during baseline).

Analysis of morphine-induced changes in ON- and OFF-cell firing indicated that the ability of morphine to activate OFF cells was significantly reduced by CCK microinjected into the RVM. An example of two OFF cells recorded during administration of morphine is shown in Fig. 3. Morphine failed to produce a strong activation of the OFF cell recorded in the animal in which CCK was microinjected into the RVM, whereas that recorded following RVM saline showed a pronounced increase in activity and elimination of the OFF-cell pause. Quantitative analysis of group data demonstrates that the ongoing activity of OFF cells showed a significant increase in control animals, whereas the small increase in CCK-treated animals was not significant (Fig. 4A). The TF-related pause was also attenuated by morphine in a dose-related fashion in control animals (Fig. 4B) but not altered in CCK-treated ani-
In contrast to the attenuation of morphine activation of OFF cells, CCK did not significantly alter the responses of ON cells to morphine administration. As shown in the examples in Fig. 5, ongoing activity was depressed by morphine in both CCK-treated and control animals with no significant difference between the CCK-treated and control groups (Fig. 6). Analysis of the TF-related burst was less clear-cut because of the wide variation in the amplitude of the burst in baseline, but as shown in Fig. 6 both CCK-treated and control groups displayed a similar trend of a dose-related decrease in the number of spikes associated with the TF (or at the mean time of the baseline TF in those animals in which the TF was inhibited following morphine).

FIG. 5. CCK does not prevent the suppression of spontaneous ON-cell firing by systemically administered morphine. Top: ratemeter record shows the effect of focal application of CCK within the RVM (10 ng/200 nl) on the ability of morphine (0.75 mg/kg iv) to suppress ON-cell firing. CCK by itself had no effect on cell discharge or tail flick latency. Following morphine, ongoing activity was almost completely eliminated, although the TF was not inhibited. TF-related firing was reduced by 68% compared with baseline. Bottom: ON-cell firing was completely inhibited in an animal in which saline was microinjected into the RVM prior to systemic administration of the same dose of morphine. (1-s bins,  trials on which a flick occurred;  trials on which no flick occurred before 10-s cutoff time.)

DISCUSSION

CCK is found in neuronal processes throughout the RVM, where its distribution overlaps that of enkephalin (Skinner et al. 1997). This peptide has been shown to have excitatory effects in a number of brain regions (Boden and Woodruff 1994). Since morphine and 6-opioid agonists depress ON-cell firing but increase that of OFF cells, it seemed that CCK applied within the RVM might interfere with the analgesic effects of systemically administered morphine and that the mechanism would involve an activation of ON cells that would counteract opioid inhibition of these neurons. However, the suppression of ON-cell firing by morphine was not significantly attenuated by microinjection of CCK into the RVM, although the analgesic actions of the opiate were significantly reduced. Rather the peptide prevented opioid activation of OFF cells at a dose that had no effect by itself on the ongoing firing of the neuron.

Our findings thus add further weight to existing evidence indicating that the RVM is necessary for the analgesic actions of systemically administered morphine and demonstrate conclusively that the relevant opioid effect is activation of OFF cells (Heinricher et al. 1999; Mitchell et al. 1998; Proudfit 1980a,b; Valverde et al. 1996; Yaksh et al. 1977; Young et al. 1984). Moreover the fact that CCK prevents opioid activation of OFF cells without affecting inhibition of ON cells has important implications for the organization of the pain modulating circuitry within the RVM. Opioids do not activate OFF cells but increase that of ON cells, which are directly responsive to 6-opioid agonists, are GABAergic inhibitory interneurons. In this view, inhibition of ON cells by morphine is responsible for disinhibition of OFF cells, which in turn produces antinociception (Fields et al. 1991; Heinricher et al. 1992). The present
shown was significant only in control animals receiving 1.5 mg/kg of MS. (MS doses
the TF (TF-related burst) showed a dose-related decrease in both CCK-treated
difference between CCK-treated animals and controls.
with significant inhibition of ongoing activity in all groups, and no significant

**FIG. 6.** A: group data confirm that focal application of CCK within the
RVM did not significantly alter morphine’s ability to inhibit ON-cell firing, with significant inhibition of ongoing activity in all groups, and no significant difference between CCK-treated animals and controls. B: firing rate at time of the TF (TF-related burst) showed a dose-related decrease in both CCK-treated and control animals. However, because of the large variability in baseline, this was significant only in control animals receiving 1.5 mg/kg of MS. (MS doses shown below the axis, *P < 0.05 compared to baseline, n = 5–8 cells/group.)

data fail to support this model insofar as OFF- and ON-cell responses to opioids were differentially modulated by CCK; OFF cells did not show a significant morphine-induced increase in activity even though ON-cell discharge was significantly depressed by the opiate. These findings are thus compatible with a more recent proposal that ON cells do not function as interneurons in RVM and that opioid-sensitive GABAergic inputs from some site outside of the RVM mediate the OFF-cell pause. μ-Opioid receptors are thus presumably located presynaptically, inhibiting release of GABA from the terminal (Heinricher et al. 1999). Since CCK by itself did not alter the firing of OFF cells (or any RVM cell class), it is possible that the target for CCK is that same afferent terminal. Alternatively, CCK could interfere with the ability of the OFF cell to respond to disinhibition, for example, by reducing recruitment of endogenous δ-opioid systems (Hirakawa et al. 1999; Kiefer et al. 1993; Nichols et al. 1995; Vanderah et al. 1996; Watkins et al. 1985a) or with the ability of OFF cells to respond to excitatory inputs (Heinricher et al. 1999). Distinguishing among these possibilities will require further analysis at the cellular level.

Interestingly, Kovelowski et al. (2000) recently showed that focal application of CCK within the RVM produced mechanical allodynia and thermal hyperalgesia. The dose employed was six times that used here, and CCK has been shown in electrophysiological studies to modulate effects of dopamine
and opioids on neuronal discharge at concentrations that by themselves have no effect with activation of the target neuron only at higher concentrations (Crawley 1991). This raises the possibility that a higher concentration of CCK would activate ON cells and that this would explain the hyperalgesia seen by Kovelowski et al. (2000). Again, further studies will be required to examine this issue.

A number of neuropeptides, among them CCK, neurotensin, dynorphin, and orphanin FQ/nociceptin, are now thought to have an antiopioid or anti-analgesic effect by an action within the RVM (Heinricher et al. 1997; Pan et al. 1997; Urban and Smith 1993). The underlying mechanisms have been elucidated at the circuitry level only for orphanin FQ/nociceptin, which profoundly inhibits all three classes of RVM neurons, thus blocking opioid activation of OFF cells and interfering with opioid antinociception (Heinricher et al. 1997). The mechanisms through which orphanin FQ/nociceptin and CCK interfere with opioid antinociception are quite different, although the ultimate target, the OFF cells, is the same. Kappa agonists microinjected into the RVM attenuate the antinociceptive action of μ-opioid agonists microinjected into the periaqueductal gray. Electrophysiological studies in vitro demonstrate that kappa receptor activation inhibits a population of RVM neurons that are not directly sensitive to opioids; these may be equivalent to OFF cells, NEUTRAL cells, or both (Pan et al. 1997). This finding suggests that dynorphin and kappa receptor agonists produce an anti-analgesic effect by inhibiting OFF cells, thus preventing μ-receptor mediated disinhibition from leading to activation. Whether this is the case, or whether there is an important role for NEUTRAL cells, remains to be demonstrated in vivo. Nevertheless it seems likely that dynorphin effects are more similar to those of orphanin FQ/nociceptin in inhibiting OFF cells than to those of CCK. Similarities and differences in the mechanisms mediating the antiopioid effects of these peptides raise the possibility that a range of neurotransmitters and neuropeptides are brought into play to fine-tune pain modulating systems under different physiological conditions.

In conclusion, a number of investigators have obtained evidence that endogenous CCK is increased in conditions of opioid tolerance and certain neuropathic pain states marked by reduced opioid analgesic efficacy, and contributes to conditioned decreases in opioid analgesia (Idanpaan-Heikkila et al. 1997b; Nichols et al. 1995, 1996; Ossipov et al. 1994; Stanfa and Dickenson 1993; Wiertelak et al. 1992; Xu et al. 1994). Our data suggest that the effects of endogenous CCK on opioid analgesia may in part be due to the ability of this peptide to regulate OFF-cell activation by these drugs.

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