Neural Basis for the Hyperalgesic Action of Cholecystokinin in the Rostral Ventromedial Medulla

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Submitted 21 April 2004; accepted in final form 18 May 2004

Heinricher, Mary M. and Miranda J. Neubert. Neural basis for the hyperalgesic action of cholecystokinin in the rostral ventromedial medulla. J Neurophysiol 92: 1982–1989, 2004. First published May 19, 2004; 10.1152/jn.00411.2004. The analgesic actions of opioids can be modified by endogenous “anti-opioid” peptides, among them cholecystokinin (CCK). CCK is now thought to have a broader, pronociceptive role, and contributes to hyperalgesia in inflammatory and neuropathic pain states. The aim of this study was to determine whether anti-opioid and pronociceptive actions of CCK have a common underlying mechanism. We showed previously that a low dose of CCK microinjected into the rostral ventromedial medulla (RVM) blocked the analgesic effect of systemically administered morphine by preventing activation of OFF-cells, which are the antinociceptive output of this well characterized pain-modulating region. At this anti-opioid dose, CCK had no effect on the spontaneous activity of these neurons or on the activity of ON-cells (hypothesized to facilitate nociception) or “NEUTRAL cells” (which have no known role in pain modulation). In this study, we used microinjection of a higher dose of CCK into the RVM to test whether activation of ON-cells could explain the pronociceptive action of this peptide. Paw withdrawal latencies to noxious heat and the activity of a characterized RVM neuron were recorded in rats lightly anesthetized with methohexitol. CCK (30 ng/200 nl) activated ON-cells selectively and produced behavioral hyperalgesia. Firing of OFF-cells and NEUTRAL cells was unaffected. These data show that direct, selective activation of RVM ON-cells by CCK is sufficient to produce thermal hyperalgesia and indicate that the anti-opioid and pronociceptive effects of this peptide are mediated by actions on different RVM cell classes.

INTRODUCTION

It is now well established that the analgesic actions of opioids can be modified by various endogenous “anti-opioid” peptides, among them cholecystokinin (CCK; see Stanfa and Dickenson 1994 and Wiesenfeld-Hallin et al. 1999 for comprehensive reviews). However, the notion that CCK is recruited to negate or balance the analgesic actions of opioids has now evolved to include a broader, pronociceptive role, for this peptide (Ossipov et al. 2003). In support of this, CCK has been shown to contribute to the enhanced nociceptive behavior in models of inflammatory or neuropathic pain, although it is generally agreed that CCK transmission plays little role in nociceptive responses in normal animals (Coudere-Civiale et al. 2000b; Friedrich and Gebhart 2003; Kamei and Zushida 2001; Kovalevski et al. 2000; Nichols et al. 1996; Urban et al. 1996; Xu et al. 1994). From this vantage point, CCK is seen as directly facilitating nociception.

This latter perspective raises the question of whether the anti-opioid action of CCK is merely a product of increased nociception (leading to a higher opioid requirement) or whether anti-opioid and pronociceptive actions of CCK are mediated by distinct neural mechanisms. A logical site at which to address this question is the rostral ventromedial medulla (RVM). The role of this region in opioid analgesia and pain modulation is well documented (Fields and Basbaum 1999; Fields et al. 1991), and functional studies show that focal application of CCK in the RVM both attenuates the antinociceptive actions of morphine (Heinricher et al. 2001; Mitchell et al. 1998) and facilitates responding on cutaneous and visceral tests of nociception (Friedrich and Gebhart 2003; Kovalevski et al. 2000).

In our previous study of CCK in the RVM, we microinjected CCK at a dose (10 ng) chosen specifically to interfere with the antinociceptive effect of a range of doses of systemically administered morphine (Heinricher et al. 2001). RVM neurons can be divided into three classes: “OFF-cells,” “ON-cells,” and “NEUTRAL cells” (Fields and Heinricher 1985). In our earlier experiments, local administration of CCK had no effect on the ongoing activity of any RVM cell class, but attenuated opioid activation of OFF-cells. OFF-cell activation is sufficient to produce antinociception and known to be critical to opioid analgesia (Heinricher et al. 1999; Neubert et al. 2004). The ability of morphine to inhibit firing of ON-cells was unaffected by CCK.

Because opioid activation of OFF-cells is indirect (Heinricher et al. 1992; Pan et al. 1990), the selective interference with opioid activation of these neurons by CCK suggested that the peptide acted presynaptically. However, CCK is also known to have postsynaptic, usually excitatory, effects in a number of CNS regions. Since there is now direct evidence that ON-cells exert a net facilitatory effect on nociception (Neubert et al. 2004), this raises the possibility that a more extensive analysis of the effects of CCK within the RVM might identify direct activation of ON-cells as underlying the pronociceptive (i.e., hyperalgesic) effects of higher doses of CCK in the RVM. In this study, we used local infusion of a higher (30 ng) dose of CCK within the RVM to test this possibility. We recorded the effect of the peptide on identified RVM neurons, and as a measure of nociceptive responsiveness, the latency of the paw withdrawal (PW) reflex to radiant heat.

METHODS

Animals and surgical preparation

All experimental procedures followed the guidelines of the Committee for Research and Ethical Issues of the International Association...
for the study of Pain and were approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University. Male Sprague-Dawley rats (250–300 g, Sasco) were anesthetized with pentobarbital (60 mg/kg ip), and a catheter was inserted into an external jugular vein for administration of anesthetic. The rat was placed in a stereotaxic apparatus, a hole drilled in the skull over the cerebellum, and the dura removed to allow placement of an electrode/microinjection cannula assembly in the medulla. Body temperature was maintained at ~37°C by a circulating water pad.

Following surgery, the anesthetic level was allowed to lighten until a PW reflex could be elicited by application of noxious heat using a feedback-controlled projector lamp focused on the blackened plantar surface of the paw. The animals were maintained in a lightly anesthetized state using a continuous infusion of methohexital (15–30 mg/kg/h iv) as previously described (Barbaro et al. 1989).

**Nociceptive testing**

PW latency was used as a measure of nociceptive responsiveness. Each trial consisted of a linear increase in temperature at ~1.8°C/s from a holding temperature of 34°C until the PW occurred or to a maximum of 52°C at 10.6 s. Trials were carried out at 5-min intervals throughout the experiment. The holding temperature obviates any concern that apparent effects on PW latency were due to changes in skin temperature.

**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 and slightly rostro-caudally 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. 1983). Spike waveforms were monitored and stored for off-line analysis (Datawave Systems, 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 PW. Cells of a third class, neutral cells, were identified by a sudden burst of activity beginning just prior to the reflex-related burst, is necessary because a burst cannot be identified unless the neuron is inactive at the time of heat onset. Cycling. The proportion of time that a given cell was considered to be in an “active” or “silent” period was defined as described previously (Barbaro et al. 1989). Briefly, an active period was defined as any epoch lasting ≥2 s with a minimum of 1 spike/s, and a silent period as any epoch of ≥2 s without any cell activity. The proportion of time in which each cell was active or silent was calculated for the baseline and postinfusion intervals in groups in which there was an overall change in ongoing activity (calculated as described above for the 30-s sample prior to each PW trial).

Data are presented as mean ± SE. Student’s t-test for correlated means was used to compare baseline and postinjection values for PW latencies and ongoing discharge if two-way repeated measures ANOVA indicated a significant overall effect of drug over time. A t-test for correlated means was also used to compare baseline and postinjection values for the on-cell burst, proportion of time active, and duration of the off-cell pause. P < 0.05 was considered statistically significant.

**Histology**

At the conclusion of the experiment, recording sites were marked with an electrolytic lesion and infusion sites by microinjection of Pontamine sky blue dye. Animals were killed with an overdose of methohexital and perfused intracardially with physiological saline following by 10% formalin. Recording/infusion sites were histologically verified and plotted on standardized sections (Paxinos and Watson 1997). Infusion/recording sites are shown in Fig. 1. RVM coordinates were 2.2 mm caudal to the interaural line, 7–9 mm below the brain surface. The RVM was defined as being comprised of the

**FIG. 1.** Histologically verified locations of recording/infusion sites in which cholecystokinin (CCK: ●) or vehicle (○) was infused into the rostral ventromedial medulla (RVM: △). CCK microinjection sites outside the RVM. VII, facial nucleus; SO, superior olive. Distance from the interaural line is indicated.

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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.

RESULTS

Microinjection of 30 ng CCK in the RVM produces thermal hyperalgesia in lightly anesthetized rats

Because previous behavioral studies of the effects of high doses of CCK in the RVM have been performed in awake animals (Friedrich and Gebhart 2003; Kovelowski et al. 2000) and because we had previously seen no effect of a lower dose of CCK (10 ng) in the RVM on nociceptive reflexes in anesthetized animals (Heinricher et al. 2001), we first determined whether 30 ng CCK would produce thermal hyperalgesia when microinjected into the RVM in lightly anesthetized animals. We established that application of 30 ng CCK within the RVM in lightly anesthetized rats produced a robust decrease in PW latency (Fig. 2). Hyperalgesia was evident within 10 min of the microinjection and lasted throughout the 45-min observation period. Infusion of saline vehicle into the RVM had no effect. Importantly, PW latency was unchanged following microinjection of CCK into the reticular formation surrounding the RVM (see Fig. 1 for locations), indicating that the site of action of the peptide was within the RVM rather than the adjacent medullary reticular formation.

CCK activates ON-cells selectively to produce hyperalgesia

Seven OFF-cells, 9 NEUTRAL cells, and 11 ON-cells were successfully recorded in experiments in which CCK was microinjected into the RVM during the recording. As shown in the examples in Figs. 3 and 4, the ongoing firing of both OFF-cells and NEUTRAL cells was unchanged following infusion of CCK or vehicle into the RVM. In addition, CCK had no effect on the duration of the OFF-cell pause or the delay from the final spike prior to the PW (pause onset) to the withdrawal (Table 1). The latency from heat onset to the beginning of the pause was shorter after CCK, which is not surprising given that the PW occurred at a shorter latency. Note that these values could be measured only on trials on which the OFF-cell under study was spontaneously active at the time of heat onset.

In contrast with OFF-cells and NEUTRAL cells, ON-cells displayed a substantial increase in ongoing activity following infusion of CCK (Fig. 5). As seen in Table 1, the overall increase in ongoing activity of these neurons reflected an increase in the proportion of time active as well as a substantial increase in firing rate during active periods. Vehicle microinjection had no effect on ON-cell firing (n = 7; Fig. 6). CCK injections outside of the RVM (n = 6; see Fig. 1 for locations) also had no effect on RVM ON-cell discharge (P > 0.05).

Firing rate of the ON-cells at the time of the PW reflex was also significantly increased (Table 1). This increase is related to the overall increase in ongoing activity, since there was rarely a discrete ON-cell “burst” following CCK (e.g., see Fig. 5). Indeed, 8 of 11 cells were already active at the time of heat onset on at least five of the six trials falling in the postinfusion analysis period. Overall, the cell under study was already active at heat onset on only 42.4% of baseline trials but on 84.8% of post-CCK trials. This increase in the probability of being in an active period at the time of the PW trial precluded analysis of the burst onset or duration. Vehicle microinjection had no effect on reflex-related firing rate (13.3 ± 2.4 sp/s at the
time of the reflex in baseline, 14.5 ± 2.7 sp/s after saline, \( P > 0.05 \). Injections adjacent to the RVM were similarly ineffective (17.3 ± 5.2 sp/s at the time of the reflex in baseline, 21.5 ± 7.1 sp/s after saline, \( P > 0.05 \)).

The effects of 30 ng CCK in the RVM on the ongoing firing of ON- and OFF-cells and NEUTRAL cells are summarized in Fig. 7, which shows ongoing firing rate before and after CCK infusion for all three cell classes.

**DISCUSSION**

At low doses, microinjection of CCK within the RVM blocks morphine-induced activation of OFF-cells and attenuates the analgesic effect of the opioid (Heinricher et al. 2001; Mitchell et al. 1998). However, at higher doses, CCK produces behaviorally measurable hyperalgesia in awake animals (Friedrich and Gebhart 2003; Kovelowski et al. 2000). The principle finding of this study is that focal administration of a higher dose, 30 ng, of CCK in the RVM activates ON-cells selectively, producing thermal hyperalgesia. Firing of OFF-cells and NEUTRAL cells is unaffected. These data thus confirm previous behavioral observations in awake animals and point to activation of ON-cells as the mechanism underlying the hyperalgesic action of this peptide.

**Role of CCK in descending control of nociception**

The anti-opioid effects of CCK are well documented, although whether CCK also modulates “nonopioid” analgesia, having a more general “anti-analgesic” effect, is unknown. Administration of CCK agonists can diminish opioid analgesia (Faris et al. 1983; Li and Han 1989; O’Neill et al. 1989), whereas CCK antagonists or CCK2 receptor antisense treatment enhances the antinoceptive effects of exogenous and endogenous opioids. CCK blockade can also slow or prevent the development of opioid tolerance in some paradigms (Chapman et al. 1995; Dourish et al. 1990; Hoffmann and Wiesenfeld-Hallin 1994; Maldonado et al. 1993; Mitchell et al. 2000; Watkins et al. 1984, 1985a,b). In addition, CCK is implicated in environmentally induced changes in nociception (Chen et al. 1998; Hendrie et al. 1989; Lavigne et al. 1992; Wiertelak et al. 1992, 1994). Moreover, there is evidence that alterations in the availability of CCK contribute to the changes in analgesic potency of opioids seen in conditions of stress, inflammation, and some pathological pain states (Coudere-Civiale et al. 2000a; Friedrich and Gebhart 2000; Hawrasko and Smith 1999; Nichols et al. 1995; Stanfa and Dickenson 1993). Although the focus of much of the work concerned with CCK-opioid interactions has been at the level of the spinal cord, specific brain regions, including the RVM, have been implicated in this anti-opioid action of CCK (Heinricher et al. 2001; Kovelowski et al. 2000; Mitchell et al. 1998; Pu et al. 1994; Tortorici et al. 2003; Watkins et al. 1985b). We previously showed that infusion of a low dose of CCK (10 ng) into the RVM prevents the analgesic effect of systemically adminis-
The RVM activates ON-cells selectively to produce behavioral finding that local application of a higher dose of CCK within underlying the anti-opioid effects of the peptide. The present recruitment of additional neural mechanisms distinct from those raised the question of whether this hyperalgesia requires re-

states (Friedrich and Gebhart 2003; Kovelowski et al. 2000)

to hyperalgesia seen in in
dently or in parallel. However, parallel recruitment seems

likely, since opioids are known to induce CCK release and prolonged opioid administration can actually facilitate nocicep-
tion (de Araujo Lucas et al. 1998; Gardell et al. 2002; Gustafsson et al. 1999; Herman et al. 2002; Larcher et al. 1998; Lucas et al. 1999; Ossipov et al. 2003; Simonnet and Rivat 2003; Vanderah et al. 2001a, b).

The combined microinjection/recording approach employed here made administration of additional drugs impractical. We were thus unable to use selective antagonists to determine the CCK receptor type involved. However, both anti-opioid and pronociceptive actions of CCK are generally attributed to the CCK₂ (CCK-B) receptor (Ossipov et al. 2003; Wiesenfeld-Hallin et al. 1999). The dose-dependence of the behavioral and neuronal effects of the peptide is therefore not likely due to an action of the peptide at different receptors. It may be that the receptor mediating the CCK block of opioid activation of off-cells is more easily accessed when exogenous peptide is given by microinjection.

Role of ON- and OFF-cells in pain modulation

The combined microinjection/single cell recording approach allows us to manipulate the firing of RVM neurons and determine the effects of that manipulation on nociceptive responding. The present observation that direct, selective activation of RVM ON-cells by local application of CCK leads to thermal hyperalgesia adds to a growing body of correlative and direct evidence that ON-cells facilitate nociception, at least as measured by a withdrawal reflex to noxious heat (Bederson et al. 1990; Foo and Mason 2003; Heinricher et al. 1989; McCagraughty et al. 2003; Neubert et al. 2004; Ramirez and Vanegas 1989). The conclusion that ON-cells facilitate nociception is also consistent with recent findings of Burgess et al. (2002) and Porreca et al. (2001), who have shown that μ-opioid receptor-expressing neurons, likely to be ON-cells (Heinricher et al. 1992), are required for allodynia and hyperalgesia following spinal nerve ligation. Notably, the influence of the ON-cell output is sufficiently potent to produce this behavioral effect in the absence of concomitant or preexisting sensitization of dorsal horn circuitry, as presumably occurs in inflammatory or nerve injury models (Mansikka and Pertovaara 1997; Pertovaara 1998; Porreca et al. 2002; Urban and Gebhart 1999). Whether these neurons also modulate responding to noxious mechanical or visceral stimulation will require testing of the effect of experimental manipulation of their firing rate or pattern on behavioral responses to mechanical or visceral stimulation.

RVM mechanisms for hyperalgesia mediated by ON-cells include a reduction in the threshold at which the ON-cell burst is triggered or a modification of the pattern of ongoing activity so that the ON-cell population is more likely to be active at the onset of the noxious stimulus (Heinricher et al. 1989; McCagraughty et al. 2003; Neubert et al. 2004). The primary change following administration of CCK was a shift in the firing pattern of ON-cells, such that this population was more likely to be active at any given time following the microinjection. As a consequence, the noxious heat stimulus, which was delivered to the paw at constant 5-min intervals, was more likely to fall at a time when the ON-cell population was active and OFF-cells were inactive. The reflex response to the heat therefore occurred at a shorter latency.

**FIG. 7.** Mean ongoing firing of ON-cells, OFF-cells, and NEUTRAL cells in baseline compared with the postinjection period in animals microinjected with 30 ng CCK (left) or saline vehicle (right) in the RVM. Only ON-cells show a significant effect of CCK. OFF-cell and NEUTRAL cell discharge is unchanged. Baseline activity was comparable between CCK and vehicle groups for each cell class. *P < 0.05, discharge following CCK compared with that in baseline.

**TABLE 1.** The effect of CCK on ongoing firing rate of ON-cells, OFF-cells, and NEUTRAL cells in baseline compared with the postinjection period in animals microinjected with 30 ng CCK or saline vehicle. Only ON-cells show a significant effect of CCK. OFF-cell and NEUTRAL cell discharge is unchanged. Baseline activity was comparable between CCK and vehicle groups for each cell class. *P < 0.05, discharge following CCK compared with that in baseline.
Only ON-cells showed a change in discharge following microinjection of CCK in the RVM. Activation of these neurons is thus either a direct postsynaptic effect of the peptide on these neurons or a presynaptic effect on a synaptic input. Iontophoretic experiments will be required to determine whether ON-cells are directly sensitive to CCK, which would distinguish between these possibilities. However, in either case, the net effect is to activate ON-cells selectively and produce thermal hyperalgesia.

Initial concepts of RVM circuitry proposed that ON-cells served as inhibitory interneurons within the RVM and that they mediated the reflex-related off-cell pause (Fields and Heinricher 1985). This hypothesis was based on a number of characteristics of the two cell classes. First, ON-cells, by definition, show a burst of activity at the time of the withdrawal reflex, that is, at the time of the off-cell pause. Second, opioid activation of off-cells is mediated by disinhibition, whereas ON-cells are known to be directly sensitive to mu-opioid agonists (Heinricher et al. 1994; Pan et al. 1990). Third, spontaneous firing of ON- and off-cell populations is out of phase (Barbaro et al. 1989). However, recent observations are inconsistent with the idea that ON-cells inhibit OFF-cells. We recently showed that disinhibition of ON-cell firing does not result in increased off-cell discharge (Heinricher and McGaraughty 1998) and that neurotensinergic activation of ON-cells does not suppress off-cell firing (Neubert et al. 2004). The present finding that selective activation of ON-cells by CCK has no effect on off-cell firing extends these observations and confirms the idea that the ON- and off-cell outputs can be controlled independently.

The classification of RVM neurons into ON-, OFF-, and neutral cells as used by us has been corroborated by the consistent pharmacology within each class, including responses to mu- and delta-opioids, alpha2-adrenergic agonists, neurotensin, and cannabinoids, as well as block of endogenous excitatory amino acid inputs by N-methyl-d-aspartate (NMDA) and non-NMDA receptor antagonists (Harasawa et al. 2000; Heinricher and McGaraughty 1998; Heinricher et al. 1988, 1992; Meng et al. 1998; Neubert et al. 2004). The present finding that CCK acts uniformly and selectively to activate ON-cells further validates the relevance of the physiological classification.

Technical considerations

There are a number of technical issues that must be considered in any study using the microinjection technique. Diffusion of drugs to sites distant from the intended target is a primary concern. That seems unlikely to be an issue in these experiments because missed placements in regions adjacent to the RVM had no effect on PW latency. A second consideration is pharmacological specificity. However, the hyperalgesia seen in this study is entirely consistent with thermal hyperalgesia and mechanical allodynia reported by Kovelowski et al. (2000) in awake animals. The differential effect of CCK on ON-cells, the association of behavioral and neuronal changes, and the lack of effect of the vehicle also argue against a nonspecific effect of the peptide. Finally, it must be recognized that there may be RVM neurons not accessible to extracellular recording, and such neurons may be responsible for the behavioral effects seen here. However, the alterations of ON-cell firing are clearly sufficient to explain the behavioral changes, and activation of this cell class remains the most parsimonious explanation of the behavioral effects of CCK.

In conclusion, these data, together with previous work (Heinricher et al. 2001), show that the anti-opioid and pronociceptive effects of CCK within the RVM are mediated by distinct circuits. The anti-opioid action of this peptide can be explained by its ability to block opioid activation of off-cells, the inhibitory output neuron of the RVM. In contrast, the pronociceptive action is mediated by activation of ON-cells. These data thus confirm the proposal that ON-cells are the facilitating output of the RVM and raise the possibility that anti-opioid and pronociceptive aspects of descending modulation could be recruited independently.

GRANTS

This work was supported by National Institute of Drug Abuse Grant DA-05608.

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