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

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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 methohexital. 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 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 were identified by a sudden burst of 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 occurrence of the PW. Cells of a third class, **NEUTRAL cells**, were identified by a sudden burst of activity beginning just prior to the occurrence of the PW. **ON-cells** and **OFF-cells** often show irregular alternations between periods of silence and activity, cell activity integrated over the 30 s prior to each PW trial was used as an overall index of ongoing firing. 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 followed by 10% formalin. Recording/infusion sites were histologically verified and plotted on standardized sections (Paxinos and Watson 1997). Infusion/recordings 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

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

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**FIG. 2.** Microinjection of CCK (30 ng) within the RVM produces thermal hyperalgesia. CCK (30 ng in 200 nl) produced a significant decrease in paw withdrawal (PW) latency when microinjected into the RVM. Vehicle (200 nl) had no effect nor did injections outside of the RVM (primarily dorsal and rostral; CCK out). There was no difference among the groups in baseline latencies (ANOVA; *P < 0.01 compared with baseline, t-test for correlated means). Neurons were successfully recorded in 26 of 39 animals in the CCK-RVM group, 16 of 17 animals in the vehicle group, and 12 of 18 animals in the CCK placement control group.

**FIG. 3.** Ratemeter records show lack of effect of CCK on the ongoing discharge of 2 NEUTRAL cells in the RVM. As was the case with OFF-cells, firing pattern and rate were unaffected by CCK. PW latencies decreased to 71 (top) and 77% (bottom) of these animals’ respective baselines. Triangles indicate PW trials, 1-s bins.

**FIG. 4.** Ratemeter records show lack of effect of CCK on the ongoing discharge of 2 OFF-cells in the RVM. In both cases, firing pattern and rate were completely unaffected by CCK. PW latencies decreased to 71 (top) and 77% (bottom) of these animals’ respective baselines. Triangles indicate PW trials, 1-s bins.
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 antinociceptive 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 Wiesnfeld-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; Hawranko 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-

FIG. 5. Ratemeter records show effects of 30 ng CCK on ongoing discharge of identified on-cells in the RVM. Top: dramatic activation of an on-cell following microinjection of CCK in the RVM. This neuron had little spontaneous activity in baseline (silent 81.4% of the baseline period), and firing rate during active periods was 4.8 sp/s. The cell became continuously active after CCK, and mean firing rate over the 20- to 45-min post-CCK analysis period was 21.7 sp/s. PW latency decreased to 72% of baseline following CCK in this animal. Bottom: this neuron had substantial ongoing activity throughout baseline (silent only 5.8% of the baseline period), but firing rate was increased from 10.6 sp/s at baseline to 14.9 sp/s following CCK. PW latency decreased to 56% of baseline following CCK in this animal. Triangles indicate PW trials. 1-s bins.

FIG. 6. Vehicle infusion had no effect on on-cell discharge. Two on-cells recorded during infusion of saline vehicle in the RVM. Firing pattern and rate were unaltered, as was PW latency (103 and 109% of baseline latencies, in top and bottom records, respectively). Triangles indicate PW trials. 1-s bins.
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-
tended morphine by interfering with opioid activation of off-
cells (Heinricher et al. 2001). At this dose, CCK alone had no
effect on ongoing or reflex-related firing of any cell class:
off-cells, ON-cells, or neutral cells.

The more recent evidence that CCK in the RVM contributes
type 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

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The more recent evidence that CCK in the RVM contributes
to hyperalgesia seen in inflammatory and neuropathic pain
states (Friedrich and Gebhart 2003; Kovelowski et al. 2000)
raught et al. 2003; Neubert et al. 2004; Ramirez and Vanegas
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; Gustaf-
son 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 activ-
ation 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; McGa-
raughty 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; Per-
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; McGa-
raughty 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 oc-
curred at a shorter latency.
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 μ-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 McGarraughty 1998) and that neurotransmitter 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 μ- and δ-opioids, α2-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 McGarraughty 1998; Heinricher et al. 1998, 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 Kovelowksi 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

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REFERENCES


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