

Corticotropin-Releasing Hormone Directly Activates Noradrenergic Neurons of the Locus Ceruleus Recorded *In Vitro*

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The neuropeptide corticotropin-releasing hormone (CRH) activates locus ceruleus (LC) neurons, thereby increasing norepinephrine levels throughout the CNS. Despite anatomical and physiological evidence for CRH innervation of the LC, the mechanism of CRH-evoked activation of LC neurons is unknown. Moreover, given the apparent absence of mRNA for CRH receptors in LC neurons, the exact location of action of CRH within the cerulear region is debated. Using *in vitro* intracellular recordings from rat brainstem, we examined whether CRH exerts a direct effect on LC neurons and which ionic currents are likely affected by CRH. We demonstrate that CRH dose-dependently increases the firing rate of LC neurons through a direct (TTX- and cadmium-insensitive) mechanism by decreasing a potassium conductance. The CRH-evoked activation of LC neurons is, at least in part, mediated by CRH₁ receptors and a cAMP-dependent second messenger system. These data provide additional support that CRH functions as an excitatory neurotransmitter in the LC and the hypothesis that dysfunction of the CRH peptidergic and noradrenergic systems observed in patients with mood and anxiety disorders are functionally related.

Key words: catecholamine; ceruleus; corticotropin; CRF; intracellular; norepinephrine (noradrenaline)

Introduction

Since the discovery of corticotropin-releasing hormone (CRH) as a primary initiator for ACTH release from corticotropes (Vale et al., 1981), CRH has been localized at many extra-hypothalamic sites (Swanson et al., 1983; Potter et al., 1994), where it plays an important role in the behavioral response to stress (Dunn and Berridge, 1990; Owens and Nemeroff, 1991; Valentino et al., 1993). The major source of norepinephrine (NE) in the CNS, the locus ceruleus (LC), receives CRH afferents from the brainstem nuclei paraventricularis, prepositus hypoglossi, and Barrington's and the central nucleus of the amygdala and the paraventricular nucleus of the hypothalamus (Valentino et al., 1992; Van Bockstaele et al., 1999). Some CRH afferents preferentially target the LC proper, whereas others target the rostromedial and dorsolateral pericerular regions (Van Bockstaele et al., 2001). Furthermore, CRH terminals contact LC dendrites and presynaptic terminals, thereby providing a neuroanatomical substrate for interaction between CRH and noradrenergic activity throughout the entire forebrain (Van Bockstaele et al., 1999).

CRH administered intraventricularly or locally into the LC increases the LC firing rate (FR) and NE release in its terminal

fields (Valentino et al., 1983; Smagin et al., 1995; Finlay et al., 1997; Jedema et al., 2001). CRH immunoreactivity in the LC is increased by cold exposure (Chappell et al., 1986), and CRH is functionally important for LC activation during exposure to several stressors (cold, hypovolemia, and immobilization) (Melia and Duman, 1991; Valentino et al., 1991; Smagin et al., 1997).

CRH receptors have been demonstrated in the LC (Millan et al., 1986; Primus et al., 1997; Sanchez et al., 1999). CRH receptors are divided in two main classes (Hauger et al., 2003b; Bale and Vale, 2004), and at least some receptors in the LC are of the CRH₁ receptor subtype (Sauvage and Steckler, 2001). Recent ultrastructural evidence for CRH₁ receptors on LC neurons has also been reported (Fox et al., 2002; Xu et al., 2004). CRH receptor mRNA has been detected in LC neurons of primates (Sanchez et al., 1999). However, with one exception (Zeng et al., 2003), CRH receptor mRNA has been difficult to detect in the LC of rodents (Potter et al., 1994; Chalmers et al., 1995; Van Pett et al., 2000), leading some to hypothesize a preferential presynaptic neuromodulatory role for CRH in the LC.

In addition to its site of action, the mechanism underlying the CRH activation of LC neurons is unknown. Typically, CRH receptors are coupled to G-proteins (G_s) and activate adenylate cyclase (Bale and Vale, 2004), although coupling to additional G-proteins and second messenger cascades has been reported (Grammatopoulos et al., 2001; Dautzenberg and Hauger, 2002; Blank et al., 2003). In the hippocampus, CRH inhibits a calcium-activated potassium current [$I_{K(Ca)}$] (Aldenhoff et al., 1983), whereas in the amygdala CRH activates a calcium current (I_{Ca}) and inhibits $I_{K(Ca)}$ (Rainnie et al., 1992; Yu and Shinnick-

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Gallagher, 1998). In corticotropes, CRH activates I_{Ca} and a hyperpolarization-activated current (I_H) and inhibits an inward-rectifying current [$I_{K(IR)}$] (Kuryshv et al., 1996a,b, 1997).

In the present studies, we have characterized the effect of application of CRH on the electrophysiological activity of LC neurons in horizontal brainstem slices of rats using *in vitro* intracellular recording techniques.

Materials and Methods

Animals. Male Sprague Dawley rats (Hilltop, Scottsdale, PA) were housed singly in hanging stainless steel cages in a colony room maintained at an ambient temperature of 23°C. Lights were maintained on a 12 hr light/dark cycle (lights on at 8:00 A.M.), with food (laboratory rodent diet 5001; PMI Feeds, St. Louis, MO) and water available *ad libitum*. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and were handled in accordance with the United States Public Health Service *Guide for the Care and Use of Laboratory Animals*.

Tissue preparation. Rats (180–300 gm) were anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused through the ascending aorta with an ice-cold, oxygenated (low Na/high sucrose) perfusion solution (in mM: 1.9 KCl, 1.2 Na_2HPO_4 , 6 MgCl_2 , 33 NaHCO_3 , 20 glucose, and 229 sucrose saturated with 95% O_2 /5% CO_2) (Aghajanian and Rasmussen, 1989). After decapitation, the brain was removed rapidly, placed in cold perfusion solution, and 300- μm -thick horizontal slices containing the LC were prepared using a DSK Microslicer (Ted Pella, Redding, CA). Tissue was transferred to cold, oxygenated artificial CSF (aCSF; in mM: 124 NaCl, 5 KCl, 1.2 KH_2PO_4 , 2.4 CaCl_2 , 1.3 MgSO_4 , 26 NaHCO_3 , and 10 glucose saturated with 95% O_2 /5% CO_2). After a recovery period of a minimum of 60–90 min, sections were transferred to a temperature-controlled recording chamber (RC-22C; Warner Instruments, Hamden, CT) where they were superfused with oxygenated aCSF at a flow rate of 0.8–1.5 ml/min at 35°C.

Electrophysiology. Intracellular recordings were obtained from neurons in the LC that were initially identified by their location within the *trans*-illuminated slice. Electrodes were filled with biocytin (2% biocytin in 2–3 M $\text{KC}_2\text{H}_3\text{O}_2$, KCl, or $\text{Cs}_2\text{H}_3\text{O}_2$) and had an impedance of ~50–100 M Ω . Putative LC neurons were identified based on a regular pattern of low-frequency discharge of long duration action potentials, as described in previous studies (Andrade et al., 1983; Williams et al., 1984; Ivanov and Aston-Jones, 1995), and had a minimal spike amplitude of 65 mV. Electrodes were connected to the headstage (HS-2A) of an Axoclamp-2A amplifier (Axon Instruments, Union City, CA) in current-clamp mode. The output of the amplifier was displayed and analyzed using an analog-to-digital board (Microstar Labs, Bellevue, WA) and customized data analysis software (Neuroscope). After determination of basal activity for 3–10 min, CRH (1 ng/ml solution) was locally applied by pressure ejection using a Picospritzer II (General Valve, Parker Hannifin, Fairfield, NJ) from a calibrated glass pipette positioned in the bath above the slice (tip size, 10–20 μm inner diameter; ~400–600 μm from recording site; ejection pressure, 50–75 psi). Pipettes were calibrated at the end of each experiment by measuring the distance the meniscus moved after ejection of ~50 pulses of a duration used during the experiment, and the ejection volume was calculated. For each neuron, multiple doses of CRH (average ejected volume, 143 nl per ejection per neuron) were administered to determine the sensitivity of the neuron to CRH, which appeared to be a function of the responsiveness of the individual neuron tested and extrinsic factors such as the proximity of the CRH pipette to the neuron and the fluid level in the recording chamber.

Drugs. Rat/human CRH obtained from Research Biochemicals (Natick, MA) or Bachem (Torrance, CA) was dissolved to a concentration of 1 $\mu\text{g}/\mu\text{l}$ (in ~90 μl) aCSF containing 0.1% bovine serum albumin and 0.3 mM ascorbate. Additional rat/human CRH received as a gift from Dr. J. Rivier (Clayton Foundation Laboratories for Peptide Biology, Salk Institute, La Jolla, CA) was dissolved in the same manner. In general, it was necessary to acidify the solution using 1 μl of a 30% acetic acid solution. D-Phe-CRH (12–41) and α -helical CRH were obtained from Bachem. A 1 $\mu\text{g}/\mu\text{l}$ stock solution of antagonist was prepared in aCSF

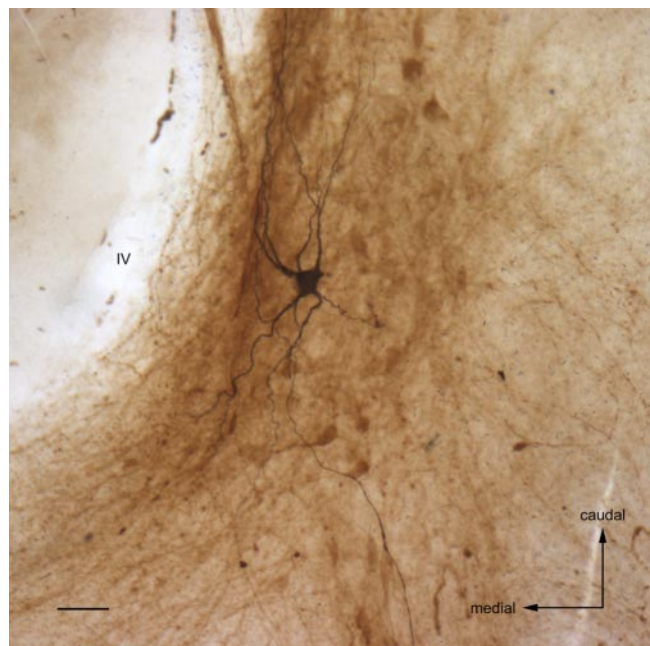


Figure 1. Identification and morphology of LC neurons. An LC neuron stained with biocytin (black) after intracellular recording in a 300- μm -thick horizontal slice of the rat brainstem is shown. Immunoreactivity for TH (brown) in somata of surrounding neurons outlines the LC. Immunoreactive processes illustrate the preservation of the extensive arborization of LC neurons outside the nucleus proper, which is where the majority of CRH innervation occurs. Scale bar, 50 μm . IV, Fourth ventricle.

containing 0.1% bovine serum albumin and 0.3 mM ascorbate. The solution was acidified using 1 μl of a 30% acetic acid solution per 100 μl of aCSF. For experiments with bath application of antagonists, the stock solution was further diluted to a final concentration with aCSF. To determine the effect of the antagonist, the effect of CRH was determined before and (at least 5 min) after bath application of the antagonist. In experiments using local antagonist administration, the stock solution of antagonist (1 $\mu\text{g}/\mu\text{l}$) was administered from a separate pipette via a second Picospritzer starting 1–10 sec before CRH administration. CP154,526, a CRH1-specific antagonist, was a gift from Pfizer (Groton, CT). A stock solution of CP154,526 was made by dissolving the compound in either 0.1 M HCl or in aCSF containing 10% DMSO. The stock solution was subsequently diluted to a final concentration using aCSF. The final DMSO concentration in the buffer was $\leq 0.1\%$. Apamin was obtained from Calbiochem (La Jolla, CA). Tetrodotoxin (TTX) and all other compounds were obtained from Sigma (St. Louis, MO). All drugs were dissolved in aCSF and bath applied at the concentration mentioned, with the exception of potassium chloride, cesium acetate, and the protein kinase A (PKA) inhibitors Rp-cAMP-S and H89 (Calbiochem, San Diego, CA), which were applied intracellularly via the recording electrode. The exchange from aCSF to drug-containing aCSF was achieved using a switch valve (Upchurch Scientific, Oak Harbor, WA). After switchover, it required ~45 sec for the drug-containing solution to reach the recording chamber and 2–3 min before stabilization of the drug effect.

Biocytin was injected into the recorded cell for histological verification. The activity and responsiveness was determined in only one neuron per slice.

Immunocytochemistry. The location of the recorded neurons was verified microscopically to be within the LC (Fig. 1), and as can be seen from the image, the use of horizontally cut sections allowed the preservation of a large part of the extensive dendritic arborization of LC neurons (Travagli et al., 1996) outside the LC proper, which included areas where the majority of CRH innervation is known to occur (Van Bockstaele et al., 2001). Immunocytochemical processing for TH and biocytin was performed as described previously (Jedema and Grace, 2003). Briefly, tissue sections were postfixed in 4% paraformaldehyde or 10% buffered forma-

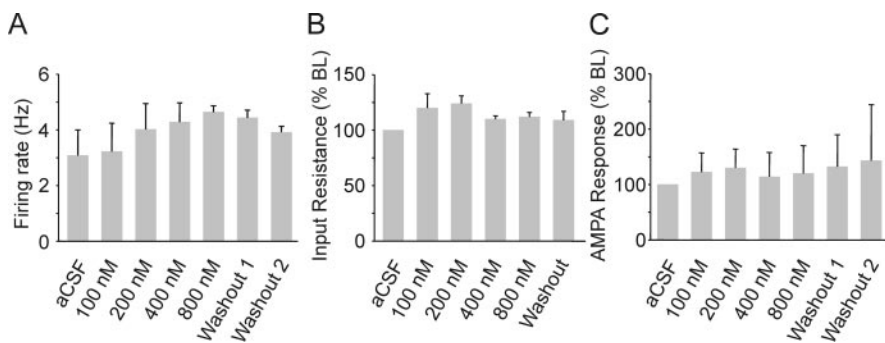


Figure 2. Bath application of CRH. *A*, Increasing concentrations of CRH increase the spontaneous discharge rate of LC neurons (maximal increase, 1.5 ± 0.9 Hz evoked by an average concentration of 325 ± 207 nM; $n = 4$ neurons). The increase in the discharge rate was variable, difficult to quantify, and required an extended period of time to wash out. *B*, Bath application of CRH increases the input resistance of LC neurons from an average of 64 ± 5 to 82 ± 10 M Ω at an average concentration of 375 ± 179 nM CRH ($n = 4$ neurons). *C*, The response to local administration of AMPA was not reliably altered by bath application of CRH. Measurements for “Washouts 1 and 2” were made between 3 and 7 min and 15 and 30 min after switchover to aCSF, respectively.

lin solution, and after a freeze–thaw procedure and overnight incubation in a 1% Triton X-100 solution, they were incubated in avidin–biotin peroxidase complex (ABC; 1:200; Vectastain Elite kit; Vector Laboratories, Burlingame, CA). The peroxidase reaction product was subsequently visualized by incubating the sections in 0.022% diaminobenzidine, 0.3% NiSO₄(NH₄)₂SO₄, and 0.003% hydrogen peroxide for 6–7 min. Then, after incubation in blocking solution containing 3% normal goat serum, 0.4% Triton X-100, and 1% bovine serum albumin, sections were incubated overnight in blocking solution containing a monoclonal antibody raised in mouse against TH (1:10,000; Chemicon, Temecula, CA), followed by incubation in secondary antibody (BA2001; 1:200; Vector Laboratories), which was then visualized using an ABC and a peroxidase reaction.

Data analysis. The membrane potential was defined as the change in the electrode potential after withdrawal of the electrode from the membrane. Action potential threshold was defined as the potential at which the greatest change in slope of the membrane potential occurred, which was determined from the second-order derivative of the membrane potential waveform. The amplitude of the action potential and afterhyperpolarization (AHP) were measured relative to this threshold, and the action potential duration was measured at the level of threshold. To determine the effect of CRH on the action potential waveform, the characteristics of all action potentials occurring during a 5 sec window around the time point of maximal CRH-evoked activation were averaged for each neuron and compared with the average of the same number of action potentials occurring immediately before CRH administration using paired *t* tests. The FR was calculated as the sliding average of the number of spikes per second occurring during a 10 sec window starting 4 sec before and ending 5 sec after the 1 sec bin for which it was calculated (Jedema et al., 2001). CRH-evoked increases in the FR were expressed as the maximal increase in the FR above baseline within 30 sec after CRH administration. We observed that this increase in the FR above baseline was fairly constant and independent of the basal FR as tested by alteration of the FR using hyperpolarizing and depolarizing current injection. The effect of application CRH antagonists or channel blockers such as TTX, apamin, BaCl₂, or CdCl₂ is compared with the CRH-evoked effect in that neuron under non-drug conditions. The average of the CRH effect before drug application and, whenever possible, after drug washout was used to calculate the reported discharge rate and action potential characteristics under control conditions. CRH-evoked depolarization was measured as the maximal change in membrane potential within 60 sec after CRH administration. The reversal potential of the CRH-evoked depolarization for each neuron was estimated by linear regression of the relationship between the maximal CRH-evoked depolarization versus membrane potential. The average basal activity calculated for the LC neurons in the present study includes the basal activity of some control LC neurons reported previously (Jedema and Grace, 2003).

Results

Basal activity of LC neurons

The vast majority of LC neurons recorded *in vitro* with potassium acetate-filled electrodes exhibited spontaneous spike firing (55 of 59), with an average basal FR of 2.2 ± 0.2 Hz. Using hyperpolarizing current steps (0.1–1.5 nA, 200 msec) injected through the recording electrode, the input resistance obtained from the slope of the current–voltage plot at the origin (*y*-intercept of the derivative of the quadratic function describing the *IV* plot) averaged 80 ± 5 M Ω ($n = 24$).

Effect of bath application of CRH

Bath application of 100, 200, 400, and 800 nM CRH increased the spontaneous FR of LC neurons, but the effect was highly variable and difficult to quantify reliably over the prolonged periods of time necessary to obtain a full dose–response curve. The maximum average increase in the discharge rate was 1.5 ± 0.9 Hz, which was achieved at an average dose of 325 ± 207 nM ($n = 4$ neurons) (Fig. 2). The input resistance was increased by bath application of CRH with a maximal increase of $31 \pm 10\%$, which occurred at an average dose of 375 ± 179 nM CRH. The response to local administration of AMPA via pressure ejection was not reliably altered by bath application of CRH. The increase in the discharge rate of LC neurons did not return to baseline within prolonged washout periods (20–30 min). Because of the variability of the results, the high expense associated with bath application of CRH, and the risks of adsorption of neuropeptide to the plastic tubing and desensitization/tachyphylaxis of the response with continuous application (Hauger et al., 2003a), we examined the effects of CRH applied locally to the neuron via a (post) calibrated micropipette with pressure ejection.

Effect of locally administered CRH on spontaneous activity

Administration of CRH (average dose, 143 ± 15 ng; average dose range, 69–236 ng) by pressure ejection transiently increased the FR of 51 of 53 neurons (Fig. 3). The increase was dose dependent with an average increase of 0.7 ± 0.1 Hz occurring $\sim 13 \pm 1$ sec after ejection of CRH from the pipette, and the CRH-evoked activation subsided by 90–120 sec after the termination of the CRH ejection. LC neurons showed little, if any, short-term desensitization in their response when pulses of CRH were administered repeatedly at ~ 3 min intervals. Similar results were obtained when the recording electrodes were filled with potassium chloride, suggesting that chloride conductances are unlikely to be involved in the effect of CRH on LC neurons. These findings are consistent with the existing evidence of an excitatory effect of CRH on LC neurons observed *in vivo* (Valentino et al., 1983, 1991, 1993; Conti and Foote, 1995; Curtis et al., 1997; Pavcovich and Valentino, 1997; Jedema et al., 2001). The concentration at the receptor site is difficult to estimate when CRH is applied via pressure ejection, particularly because the drug is washed out rapidly by fresh buffer entering the recording chamber. Based on an average volume of 143 nl per CRH administration applied over 1040 msec, one would estimate a >1000 -fold dilution in the recording chamber (200 μ l), resulting in an estimated CRH concentration of ~ 150 nM. This is within the range of concentrations used in our experiments with bath application of CRH described

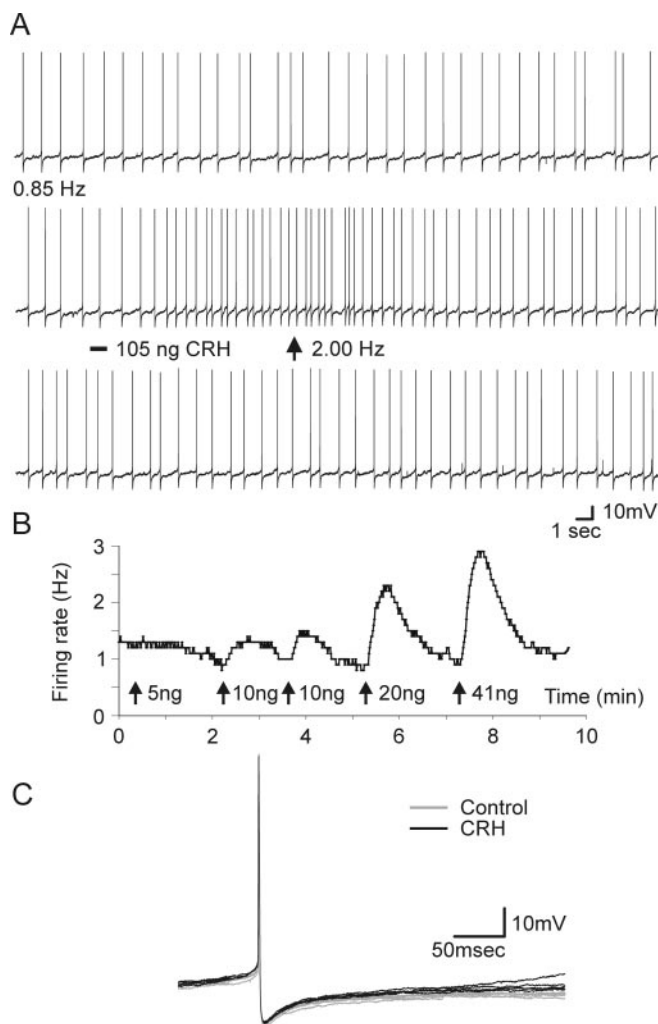


Figure 3. Local administration of CRH increases the FR of spontaneously active LC neurons by increasing the rate of repolarization. *A*, The average baseline FR of this spontaneously active LC neuron was 0.85 Hz. CRH (105 ng) was ejected (horizontal bar) in the bath $\sim 400 \mu\text{m}$ from the recording site. A maximum FR of 2.00 Hz was reached at 12 sec (arrow) after CRH ejection. *B*, Repeated CRH administration causes an increase in the FR that is transient and dose dependent. The maximal activation in this LC neuron occurs ~ 15 sec after CRH ejection, and the FR has returned to baseline levels within 90–120 sec. *C*, An overlay of five action potential waveforms at the time point of maximal CRH-evoked activation (black) demonstrates that the waveform is similar to the waveform of five action potentials from the same neuron immediately before CRH ejection (gray). At the time of maximal CRH-evoked activation, the rate of repolarization after action potential discharge is faster, leading to a more rapid return to action potential threshold and consequently an increase in the FR of the neuron.

above. In addition, in previous studies, 15–500 nM CRH has been used for electrophysiological experiments on corticotropes (Kuryshv et al., 1996a,b, 1997) and neurons in the amygdala and hippocampus (Aldenhoff et al., 1983; Siggins et al., 1985; Aldenhoff, 1986; Rainnie et al., 1992; Yu and Shinnick-Gallagher, 1998). In studies on an LC-like cell line (CATHa), 500 nM CRH was necessary to achieve a maximal effect on neurite outgrowth and cAMP accumulation (Bundey and Kendall, 1999; Thiel and Cibelli, 1999; Cibelli et al., 2001).

Effect of CRH on action potential characteristics

There was no difference in the action potential waveform of LC neurons recorded immediately before and at the point of maximal CRH-evoked activation (Table 1). However, the rate of repolarization of the membrane potential after action potential dis-

Table 1. Action potential characteristics

	Control	CRH
Threshold (in millivolts)	-45.9 ± 2.1	-45.7 ± 2.1
Amplitude (in millivolts)	71.7 ± 1.2	71.3 ± 1.2
Duration (in milliseconds)	2.0 ± 0.1	2.0 ± 0.1
AHP (in millivolts)	20.4 ± 0.5	20.3 ± 0.6
Interspike interval (in milliseconds)	585 ± 114	$^a 421 \pm 84$
Rate of repolarization (in millivolts per second)	55 ± 7	$^a 74 \pm 9$

Data show the characteristics of spontaneously occurring action potentials recorded during a 5 sec epoch of maximal CRH-evoked activation compared with the same number of action potentials recorded immediately before CRH administration (2 pulses for each neuron) in 10 LC neurons.

$^a p < 0.05$.

charge was faster after CRH administration, resulting in a more rapid depolarization to spike threshold (Table 1, Fig. 3). Given that CRH increases the LC FR, it was indeed expected that CRH affects conductances that are active during the period between action potentials rather than conductances that are selectively active during action potential discharge.

Effect of CRH after blockade of synaptic activity

When synaptic activity in the slice was prevented by bath application of $2 \mu\text{M}$ TTX, the spontaneous discharge of action potentials was altered, and the neurons began the discharge of putative calcium spikes (Williams et al., 1984). In most cases (four of six), the neurons would cease their spontaneous activity, although calcium spike discharge activity could be reinstated by injecting a small amount of depolarizing current into the neuron (20–250 pA). In two cases, these calcium spikes continued to occur spontaneously. Administration of CRH (at the same doses as before TTX) caused a transient increase in calcium spike activity with a similar time course as in the same cell tested before TTX perfusion. This was observed in six of six LC neurons (Fig. 4). The fact that the increase in the FR persisted after blockade of synaptic activity by TTX demonstrates that the effect of CRH *in vitro* is primarily mediated via a direct action on LC neurons and that fast sodium currents are likely not to be a major contributor to the excitatory effect of CRH.

When calcium-dependent transmitter release in the slice was prevented by bath application of CdCl_2 ($100 \mu\text{M}$), the basal discharge rate of LC neurons increased (1.73 ± 0.67 Hz before and 4.58 ± 1.50 Hz after switch to CdCl_2 ; $n = 4$; $p < 0.05$); this was most likely attributable to a blockade of calcium-activated potassium conductances (K_{Ca}) (Fig. 5) (Osmanovic and Shefner, 1993). CRH administration (at the same doses as before CdCl_2) continued to increase the discharge rate of LC neurons in the presence of CdCl_2 . The absolute magnitude of the CRH-evoked increase was also higher (0.50 ± 0.16 Hz before and 0.85 ± 0.21 Hz after switch to CdCl_2 ; $n = 4$; $p < 0.01$), but the time course of activation was similar as that observed under control conditions (maximal activation at 15 ± 2 sec). These observations again demonstrate that the effect of CRH *in vitro* is primarily mediated via a direct action on LC neurons and, moreover, that changes in calcium conductances are likely not to be a major contributor to the excitatory effect of CRH. Consistent with a direct excitatory effect of CRH, no spontaneous postsynaptic potentials were observed in our slices before and during CRH administration in any experiments.

Effect of CRH on hyperpolarized LC neurons

When hyperpolarizing current was delivered through the recording electrode to prevent spike discharge, the administration of

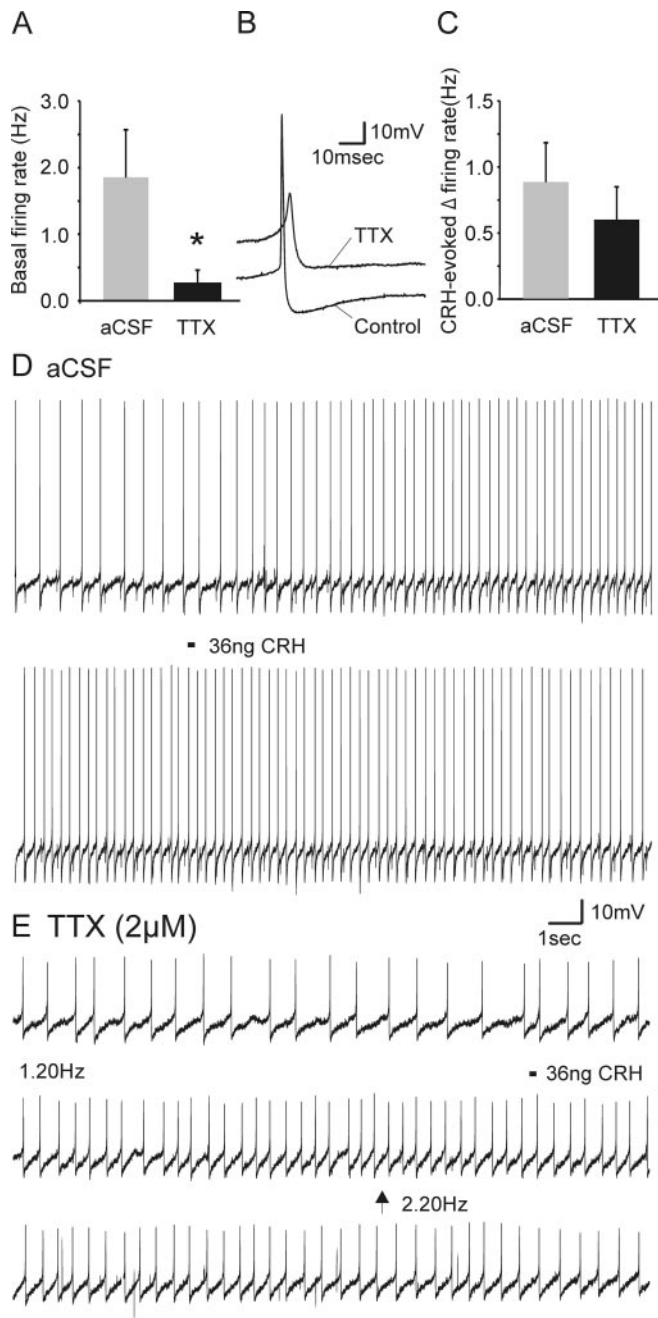


Figure 4. CRH increases the discharge rate of putative calcium spikes in LC neurons in the presence of TTX. *A*, When synaptic activity is prevented by bath application of TTX ($2 \mu\text{M}$), the basal FR of LC neurons decreases significantly. *B*, This LC neuron continued to discharge calcium spikes spontaneously. *C*, In the presence of TTX, CRH increased the calcium spike rate in six of six neurons to a similar extent as before TTX. *D*, Representative trace demonstrating that CRH administration (36 ng; horizontal bar) increased the FR of this neuron under control conditions (aCSF). *E*, In the same neuron, the calcium spike discharge rate after bath application of TTX ($2 \mu\text{M}$) was increased from 1.20 to 2.20 Hz at 16 sec after CRH administration.

CRH caused a transient depolarization (average depolarization, $2.8 \pm 0.4 \text{ mV}$) with a similar time course as the CRH-evoked increase in the FR (time point of maximal depolarization, $17 \pm 1 \text{ sec}$ after CRH) (Fig. 6). The magnitude of the depolarization was voltage dependent with an extrapolated reversal potential of $-114 \pm 8 \text{ mV}$ ($n = 21$). In some experiments ($n = 11$), repeated brief hyperpolarizing current steps (10–20 msec, 100–300 pA, 1 Hz) were applied in addition to the constant hyperpolarizing

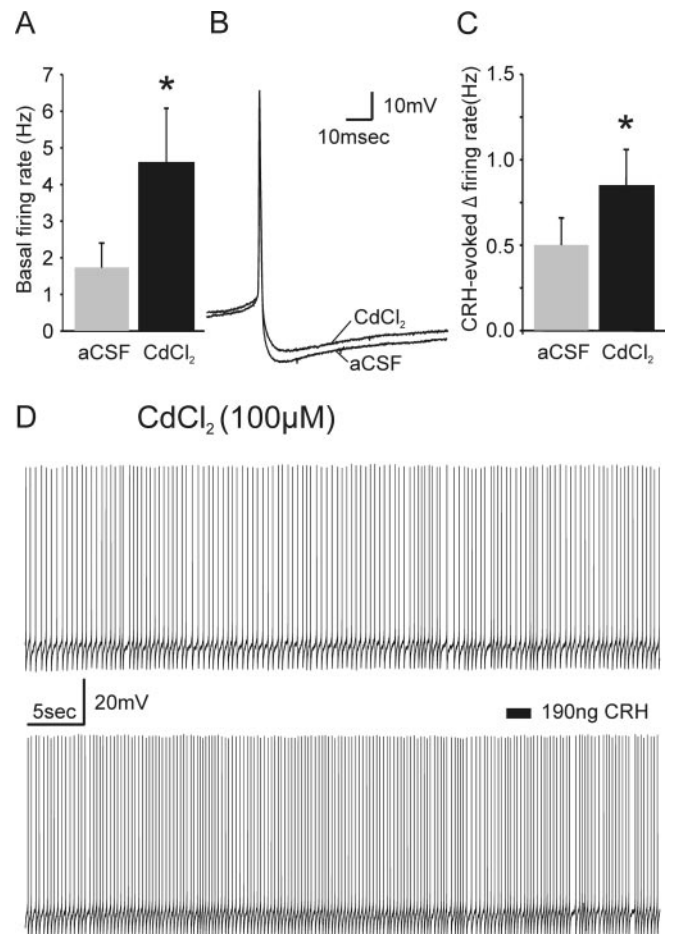


Figure 5. CRH increases the FR of LC neurons in the presence of cadmium. *A*, When calcium-dependent neurotransmitter release is prevented by bath application of the CdCl_2 ($100 \mu\text{M}$), the basal FR of LC neurons increased significantly. *B*, This increase occurs most likely as a result of blockade of calcium-activated potassium conductances, leading to a decreased AHP. *C*, In the presence of cadmium, CRH administration increases the LC FR to a greater extent (0.85 ± 0.21 vs $0.50 \pm 0.16 \text{ Hz}$; $p < 0.01$), but with a similar time course (maximal activation at $15 \pm 2 \text{ sec}$) as under control conditions. *D*, Representative trace demonstrating the CRH-evoked activation in the presence of CdCl_2 .

current to estimate changes in input resistance. An increase in input resistance was observed to coincide with the CRH-evoked depolarization (average increase, $7 \pm 1\%$ at $27 \pm 6 \text{ msec}$ after CRH ejection; $n = 11$) (Fig. 6). A depolarization of similar magnitude evoked by reducing the amplitude of constant hyperpolarizing current injected did not change the input resistance estimate (data not shown). The CRH-evoked depolarization also persisted in the presence of TTX ($n = 1$; data not shown).

Attenuation of potassium conductances

To determine whether the increase in the FR was mediated by a reduction of potassium conductances, potassium conductances were blocked with cesium applied intracellularly. As expected, LC neurons recorded with cesium-containing electrodes (3 M) exhibited a depolarized membrane potential, an increased basal discharge rate and action potential duration, and a reduced AHP (Fig. 7). Only much (approximately threefold) higher doses of CRH were able to cause an increase in discharge rate, which was significantly reduced compared with control neurons ($0.1 \pm 0.1 \text{ Hz}$; $n = 4$; $p < 0.01$). Although the response to CRH was greatly diminished, the FR of the neuron was increased by bath applica-

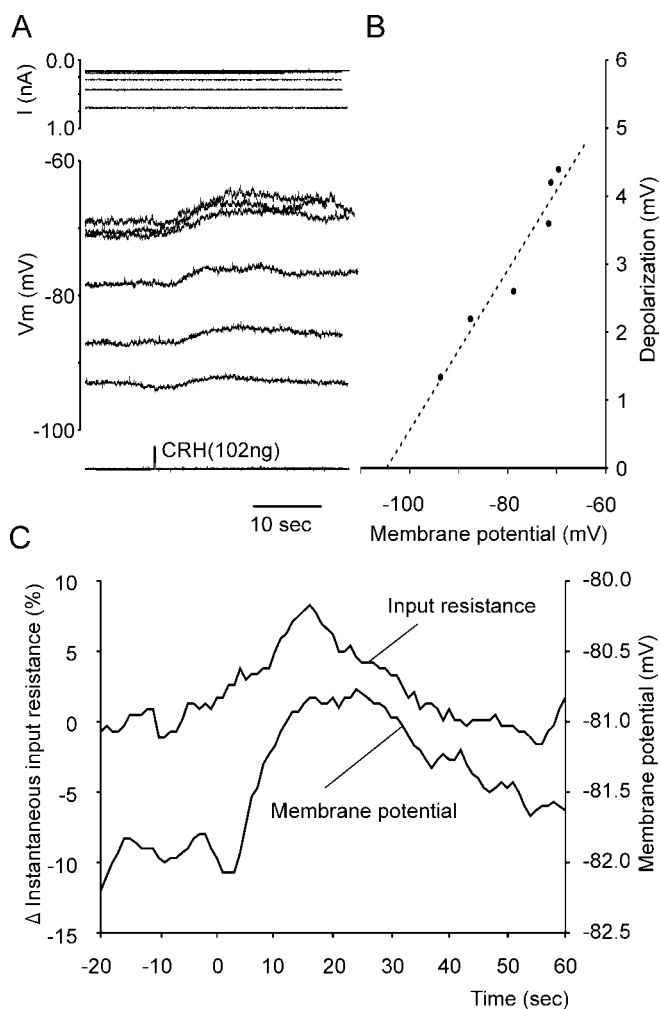


Figure 6. CRH depolarizes the membrane of LC neurons in the absence of action potential discharge. *A*, When action potential discharge is prevented by constant hyperpolarizing current delivered through the recording electrode, CRH ejection causes depolarization of the membrane potential. *B*, The magnitude of the CRH-evoked voltage deflection is voltage dependent with a reversal potential of -114 ± 8 mV ($n = 21$). *C*, The magnitude of voltage deflections produced in response to repeated hyperpolarizing current steps (0.3 nA, 10 msec, 1 Hz) increases during the CRH-evoked depolarization, indicating an increase in input resistance. Current-evoked depolarization of the same magnitude as that caused by CRH did not change the input resistance (data not shown).

tion of glutamate ($300 \mu\text{M}$). These data demonstrate that the effect of CRH on LC neurons is mediated primarily by a change in potassium conductances.

Given that in the hippocampus and amygdala the effect of CRH is thought to be mediated by altering $I_{K(\text{Ca})}$, the effect of bath application of the calcium-activated potassium channel blocker apamin (200 nM) was examined. Apamin caused a small reduction in the amplitude of the AHP; nonetheless, the CRH-evoked increase in the FR persisted during bath application of apamin in three of three neurons (Fig. 8*A*). The effect of blockade of the inward-rectifying potassium conductances [$I_{K(\text{IR})}$] on the CRH-evoked increase in the FR was examined using bath application of BaCl_2 ($100 \mu\text{M}$). BaCl_2 application increased the spontaneous FR of LC neurons in all neurons tested, and the current-voltage relationship of LC neurons during BaCl_2 application was linear, indicating that the $I_{K(\text{IR})}$ conductances were effectively blocked (Fig. 8*B*). The CRH-evoked increase in the FR persisted during bath application of BaCl_2 in five of five neurons (Fig. 8*B*).

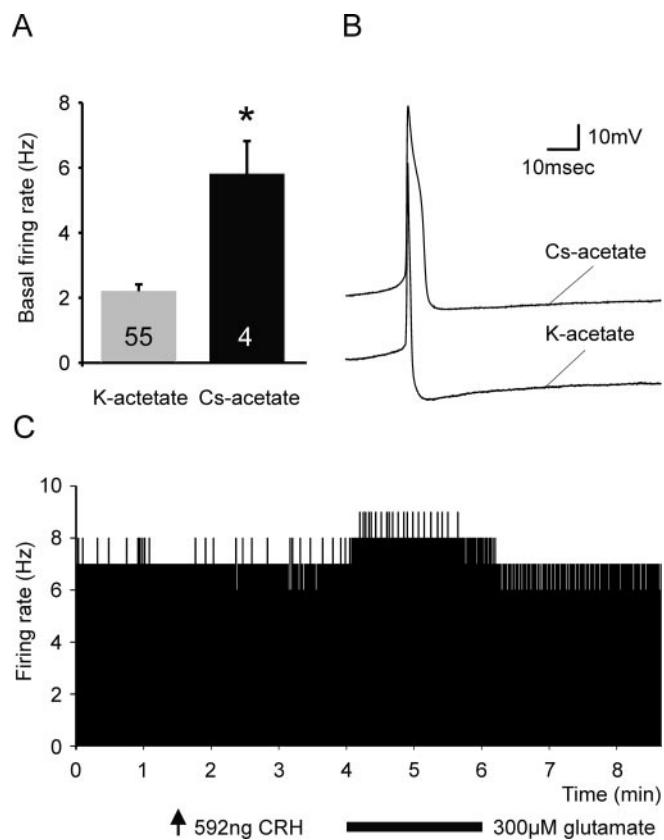


Figure 7. The CRH-evoked increase of LC neuron activity was reduced when potassium conductances are blocked by cesium. *A*, LC neurons recorded with electrodes containing cesium acetate (3 M) exhibit a higher FR. *B*, Cesium also caused a depolarized membrane potential, a much longer duration action potential, and a reduced AHP. *C*, Administration of a higher dose of CRH only causes a slight increase in the FR (0.2 Hz). Nevertheless, the FR of the neuron was increased by bath application of glutamate ($300 \mu\text{M}$).

Effect of CRH antagonists

Bath application of the CRH antagonist D-Phe-CRH ($0.1\text{--}1 \mu\text{M}$) caused a transient increase in the basal FR in five of seven LC neurons. Nevertheless, the magnitude of the CRH-evoked increase in the FR was reduced to $64 \pm 12\%$ of control by the administration of D-Phe-CRH (Fig. 9). Local application of D-Phe-CRH via pressure ejection also increased the spontaneous discharge activity. The increase in the FR elicited by local D-Phe-CRH was transient and had a similar time course as the CRH-evoked activation (Fig. 9). Vehicle ejections (acidified aCSF with 0.1% bovine serum albumin and 0.3 mM ascorbate) did not cause an activation of the LC FR (Fig. 9). Local application of another peptidergic CRH antagonist, α -helical CRH, increased the LC discharge rate in a manner similar to D-Phe-CRH. Despite the fact that local administration of α -helical CRH increased the basal FR, it reduced the absolute magnitude of the CRH-evoked activation (to $77 \pm 11\%$; $n = 3$). Bath application of the specific CRH₁ receptor antagonist CP154,526 ($1\text{--}100 \mu\text{M}$) reduced the magnitude of the effect of CRH to $66 \pm 13\%$ ($n = 7$) without alterations of the basal FR (Fig. 9).

Second messenger systems

LC neurons impaled with sharp electrodes containing the cyclic nucleotide analog and protein kinase A (PKA) inhibitor Rp-cAMP-S (4.5 mM) exhibited normal electrophysiological characteristics (spike waveform and FR) immediately after penetration by the electrode (Fig. 10). However, after a ~ 5 min delay, the

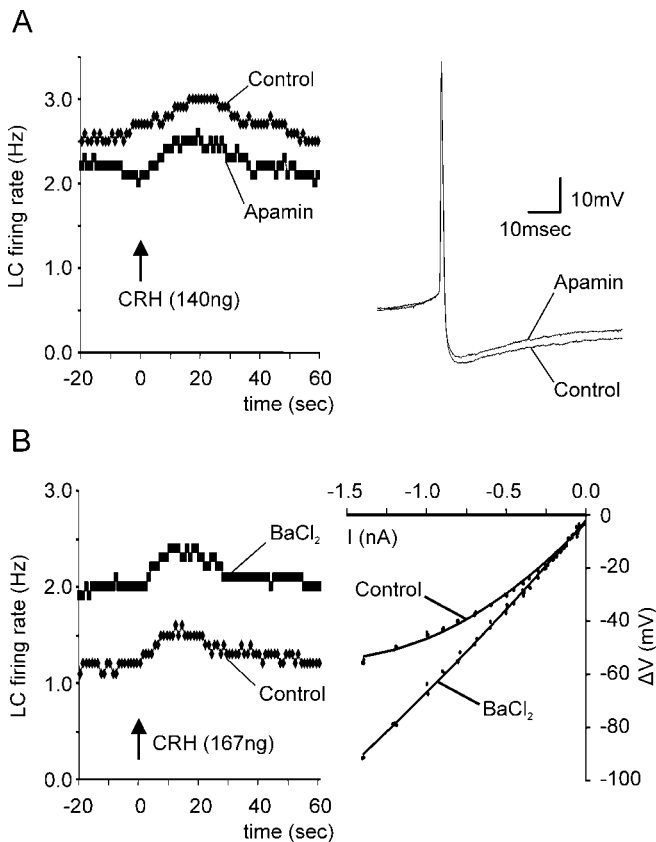


Figure 8. The CRH-evoked increase in the FR persists during blockade of several specific potassium conductances. *A*, Bath application of a blocker of calcium-activated potassium conductances, apamin (200 nM), does not affect the CRH-evoked increase in the LC FR. The effectiveness of apamin blockade on the AHP is evident in an overlay of the action potential waveform obtained before and during apamin administration. *B*, Bath application of a blocker of inwardly rectifying potassium conductances, BaCl₂ (100 μM), increases the spontaneous FR of LC neurons but does not affect the CRH-evoked increase in the LC FR. The effectiveness of barium in blocking inwardly rectifying potassium conductances is evident from a comparison of the current–voltage plots obtained before and during barium administration.

basal FR decreased and eventually ceased. When CRH was applied during the first 5 min after penetration of the neuron, an increase in activity was observed that had a similar time course as that observed in cases in which electrodes without the PKA inhibitor were used. However, at later time points, CRH was unable to increase the activity of the neurons ($1 \pm 2\%$; $n = 3$) even when much higher doses of CRH (up to 1013 ng) were tested. In experiments using intracellular administration of a different PKA inhibitor, H89 (10–50 μM), the baseline FR was also reduced (to $25 \pm 18\%$; $n = 4$); however, the CRH-evoked responses of LC neurons remained unchanged ($90 \pm 32\%$), even after prolonged periods (>45 min) to assure diffusion into the recorded neuron. These data suggest that although both PKA inhibitors decreased the FR of LC neurons, the ability of Rp-cAMP-S to attenuate the response to CRH was more likely attributable to its cAMP antagonist properties rather than PKA inhibition.

Discussion

Methodological considerations

This study used intracellular recordings with sharp electrodes. Although sharp electrodes are unlikely to achieve a good space clamp, space-clamp problems with LC neurons have been reported even in whole-cell recordings, especially when using horizontal slices (Travagli et al., 1996). We used horizontal slices to

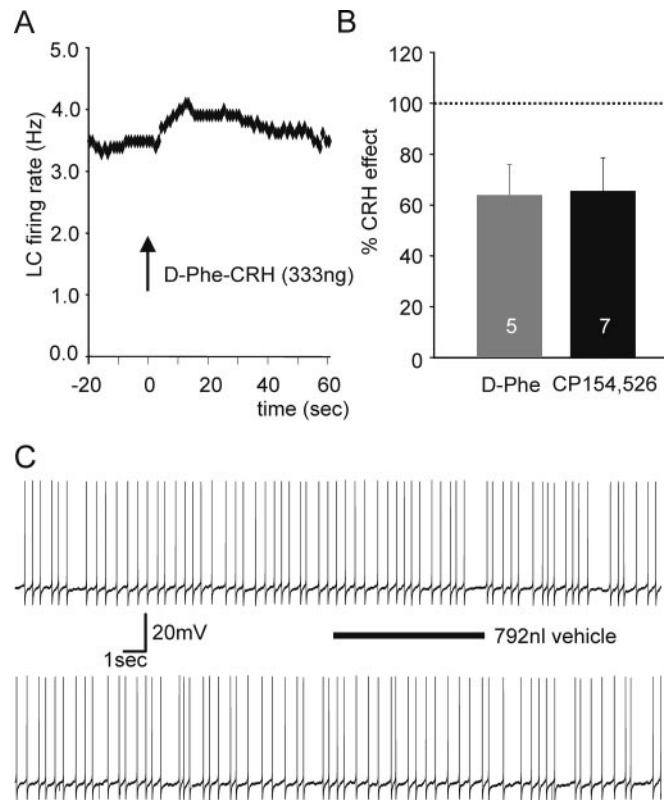


Figure 9. The magnitude of the CRH-evoked increase in the FR was reduced by CRH antagonists. *A*, Local administration of D-Phe-CRH increases the LC FR with a time course similar to the CRH-evoked activation of LC neurons. *B*, Although bath application of the CRH antagonist D-Phe-CRH (1 μM) also increased the spontaneous FR of LC neurons, it reduced the magnitude of the effect of CRH. Bath application of CP154,526 (1–100 μM) reduced the effect of CRH without increasing the basal FR. The numbers on each bar indicate the number of neurons contributing to the average. *C*, Vehicle ejection (note the large volume) does not affect the LC FR.

preserve the extracellular dendritic arborizations of LC neurons because the majority of the CRH innervation of LC neurons occurs in the pericellular region outside of the nucleus proper (Van Bockstaele et al., 1999). Washout of the intracellular messengers involved in the cAMP cascade has been reported for LC neurons (Alreja and Aghajanian, 1991, 1995). Given the reported role of cAMP for CRH function, we used sharp electrodes to confirm its role in the LC. Bath application is frequently used to permit an estimation of pharmacodynamic parameters of drugs such as ED₅₀ value. However, the response to bath-applied CRH was difficult to quantify reliably, and we chose to use local administration of CRH to avoid problems with the reported adhesion of neuropeptides to plastics and desensitization of the response to CRH as a result of the continuous presence of the peptide (Hauger et al., 2003a).

Site of action of CRH on LC neurons

Valentino and co-workers first demonstrated that CRH increases the LC FR *in vivo* (Valentino et al., 1983) and hypothesized that CRH acts within the LC given that intra-LC CRH is more potent than intraventricular CRH and that the effect of intraventricular-administered CRH and certain stressors can be blocked by intra-LC infusion of a CRH antagonist (Valentino et al., 1991; Smagin et al., 1995, 1997; Curtis et al., 1997). We demonstrate here that CRH activates LC neurons directly even when synaptic activity is prevented. This direct action is consistent with ultrastructural evidence for synaptic contacts between CRH-

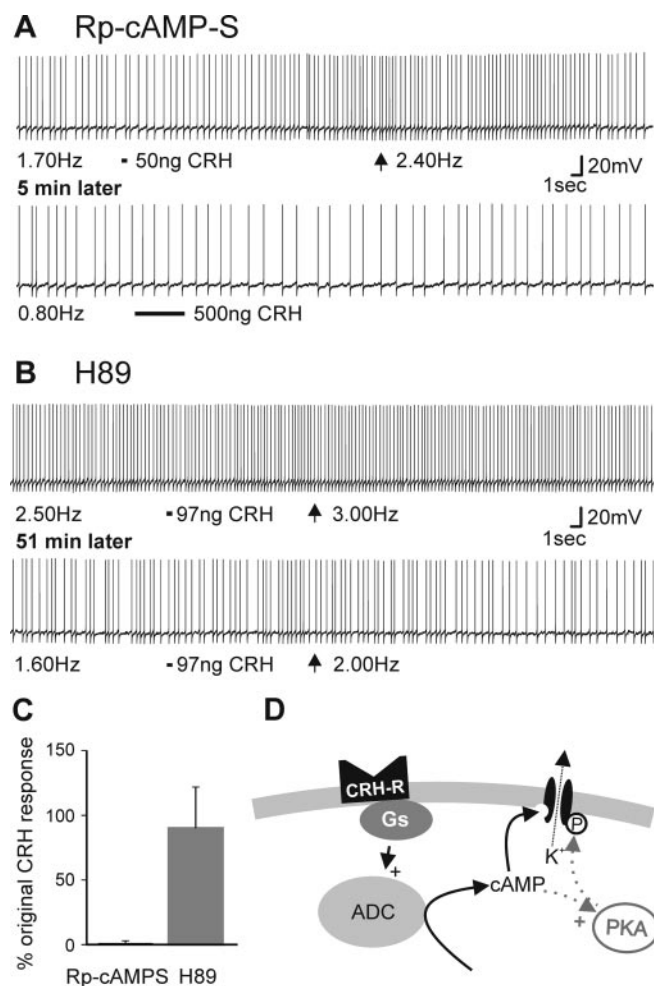


Figure 10. The increase in the LC neuron FR is prevented by intracellular administration of the PKA inhibitor Rp-cAMP-S but not by H89. *A*, When tested within the first 5 min of impalement of the neuron with an electrode containing the cyclic nucleotide analog and PKA inhibitor Rp-cAMP-S (4.5 mM; $\sim 400 \times IC_{50}$ value), CRH administration (50 ng) increased the LC neuron FR. Diffusion of Rp-cAMP-S into the cell decreases the spontaneous FR. Five minutes after the initial activation by CRH, administration of 10-fold higher doses of CRH no longer increases the LC FR. *B*, In contrast, LC neurons recorded with electrodes containing the PKA inhibitor H89 (10–50 μ M; $\sim 1000 \times IC_{50}$ value) exhibit a response to CRH that is stable over time. *C*, The increase in the LC FR in response to CRH administration at later time points was expressed as a percentage of the response to CRH immediately after entry of the neuron with electrodes containing Rp-cAMP-S ($n = 3$) or H89 ($n = 4$). *D*, We interpret these data to indicate that CRH activates LC neurons via a cAMP-dependent mechanism independent of PKA.

immunoreactive terminals and LC dendrites and by the presence of CRH₁ receptors on LC neurons (Van Bockstaele et al., 1999; Fox et al., 2002). Our *in vitro* studies do not, however, rule out the possibility of additional presynaptic actions of CRH *in vivo*. Others have argued, based on the slower time course of CRH compared with glutamate, that CRH increases NE efflux through an indirect mechanism (Palamarhouk et al., 2000, 2002). However, based on the caudal injection sites in the LC in their study, CRH would have to diffuse a much greater distance to its receptor given the point of CRH innervation in the pericerular area (Van Bockstaele et al., 1999); in contrast, infusions of glutamate should act on glutamate receptors located at multiple sites along the dendritic tree (Van Bockstaele and Colago, 1996). Moreover, the faster diffusion characteristics for glutamate based on its lower molecular weight (Nicholson and Tao, 1993) and hydrophilic nature, coupled with its very short extracellular half-life because

of an efficient uptake system (Danbolt, 2001), could further contribute to the difference in time course of glutamate- and CRH-evoked NE efflux. The fact that in the present experiments the CRH-evoked activation reaches a maximum within ~ 15 sec after local CRH administration, much faster than the 11–16 min that these authors observed, further suggests that differences in diffusion kinetics underlie the difference in the reported time course of NE efflux.

Potential mechanism of CRH action: potassium conductances

The CRH-evoked activation of LC neurons persisted in the presence of TTX and CdCl₂ but was prevented by intracellular cesium administration. Moreover, CRH administration depolarized LC neurons when spike discharge was prevented by hyperpolarizing current injection. The reversal potential of the CRH-evoked depolarization was more hyperpolarized than the reversal potential for potassium predicted by the Nernst equation (-88 mV at 35°C and $[K^+]_o = 6.2$ mM (Egan et al., 1983) and was accompanied by an increase in input resistance. The combination of an increase in input resistance and a -115 mV reversal potential suggests that a decrease in potassium conductances mostly mediates the CRH-evoked depolarization. The fact that CRH likely acted on sites electrotonically distal to the soma could account for the more hyperpolarized reversal potential for the CRH-evoked depolarization compared with that for potassium. LC neurons in general and LC neurons in horizontal slices in particular are known not to be electrotonically compact (Travagli et al., 1996). Given that a large part of the CRH innervation of LC neurons occurs in the pericerular area (i.e., distal to the soma), the reversal potential of a CRH effect measured at the soma likely overestimates the reversal potential of CRH responses at electrotonically distal sites. However, other conductances with a positive reversal potential could contribute to the CRH-evoked depolarization, resulting in a reversal potential more negative than that for potassium (Brown et al., 1971). Thus, the closing of an inward cation conductance in addition to the opening of a potassium conductance has been suggested for the enkephalin- and muscarine-mediated hyperpolarization of LC neurons (Shen and North, 1992a; Alreja and Aghajanian, 1994), whereas the opposite has been suggested for the substance P (SP)-mediated depolarization of LC neurons (Shen and North, 1992b; Koyano et al., 1993). Although in the present experiments the contribution of other conductances to the CRH-evoked depolarization cannot be completely excluded, blockade of fast sodium or calcium conductances did not prevent the CRH-mediated increase in the discharge rate of LC neurons. Therefore, the present data suggest that the majority of the CRH-evoked response is mediated by an attenuation of a potassium conductance.

The effect of CRH on LC neurons was not blocked by apamin, a specific blocker for $I_{K(Ca)}$ that is effective at blocking the intermediate component of the AHP in LC neurons (Osmanovic et al., 1990) and reduced the AHP amplitude in the present experiments. The lack of $I_{K(Ca)}$ involvement is consistent with the persistence of the excitatory effect of CRH in the presence of CdCl₂ and the depolarizing action of CRH in hyperpolarized, non-discharging neurons. The blockade of $I_{K(IR)}$ did not prevent the effect of CRH on LC neurons although barium readily blocked the inward rectification. Collectively, these data suggest that CRH modulates LC neuron activity via a different potassium conductance than has been suggested for corticotropes and neurons in the hippocampus and amygdala (Siggins et al., 1985; Aldenhoff, 1986; Rainnie et al., 1992; Kuryshv et al., 1997).

Several neurotransmitters modulate potassium conductances

to affect LC activity. Thus, neuropeptide Y (NPY), galanin, somatostatin, enkephalin, and NE reduce LC activity by increasing an inwardly rectifying potassium conductance (Williams et al., 1985, 1988; Inoue et al., 1988; Illes et al., 1993; Pieribone et al., 1995), whereas hypocretin, SP, and VIP increase LC activity by decreasing a potassium conductance (Wang and Aghajanian, 1990; Koyano et al., 1993; Ivanov and Aston-Jones, 2000). It has been suggested that enkephalin, NE, SP, somatostatin, and NPY affect the same potassium conductance (Aghajanian and Wang, 1987; Illes and Regenold, 1990; Velimirovic et al., 1995). Although the effect of somatostatin and enkephalin can be prevented by bath application of barium, it is possible that CRH may be exerting its primary effect via actions on a similar potassium conductance.

Antagonists of CRH receptors

The effect of CRH administration was partly blocked by local or bath application of the CRH receptor antagonist D-Phe-CRH. Because D-Phe-CRH is a competitive antagonist, local application of small volumes of agonist in high concentration may have effectively displaced a sufficient amount of antagonist to elicit some excitatory effect. In contrast, we noted a significant activation of LC neurons by local or bath application of the antagonist alone, with a similar time course of activation as the CRH-evoked activation. Such data are consistent with a partial agonist action of D-Phe-CRH, perhaps similar to the effect of higher concentrations of D-Phe-CRH used for raphe neurons (Kirby et al., 2000) but not in the LC (Curtis et al., 1994, 1999). However, a partial agonist effect of D-Phe-CRH in the LC could potentially be masked by a low baseline CRH tone *in vivo*. Partial agonist effects of α -helical CRH have been reported previously *in vitro* (Rainnie et al., 1992; Yu and Shinnick-Gallagher, 1998; Smart et al., 1999) and *in vivo* (Menzaghi et al., 1994; Borsody and Weiss, 1996). Nonetheless, the CRH antagonist still reduced the magnitude of the CRH-evoked increase in the LC FR. The possibility of a novel type of CRH receptor should be considered (Bittencourt and Sawchenko, 2000; Van Pett et al., 2000), although previous *in vivo* studies suggest that the receptor mediating the effect of CRH in the LC can be blocked by D-Phe-CRH (Curtis et al., 1994, 1997). The receptor subtype-specific antagonist CP154,526 did not cause a change in the basal FR but, because of its poor solubility, we could not evaluate whether the CRH effect could be completely blocked by this antagonist, and no clear dose–response relationship was observed with increasing doses of CP154,526. Nevertheless, CP154,526 did reduce the effect of CRH, suggesting that at least part of the response occurred via CRH₁ receptors; this is consistent with recent evidence demonstrating CRH₁ receptor immunoreactivity in the LC (Sauvage and Steckler, 2001; Fox et al., 2002; Xu et al., 2004) and the observation that systemic CP154,526 reduces intraventricular CRH-evoked increases in the LC FR (Schulz et al., 1996).

Second messenger systems

Consistent with previous reports that cAMP and PKA are necessary for spontaneous activity of LC neurons (Alreja and Aghajanian, 1991, 1995), intracellular administration of the cAMP antagonist/PKA inhibitor Rp-cAMP-S and the PKA inhibitor H89 both reduced the spontaneous discharge rate of LC neurons. However, only Rp-cAMP-S prevented the CRH-evoked activation of LC neurons. Both compounds, which have comparable molecular weights, were applied intracellularly through the recording electrode at sufficient concentrations (~400-fold and 1000-fold K_i for Rp-cAMP-S and H89, respectively) because they

both decreased LC neuron discharge. Direct actions of cAMP on cellular conductances, such as I_H or cyclic nucleotide-gated channels, can occur independent of PKA (Pedarzani and Storm, 1995; Ingram and Williams, 1996; Chin et al., 2002). These actions can be prevented by cAMP antagonists, such as Rp-cAMP-S, but not by PKA inhibitors. Therefore, it appears that the effect of CRH on LC neurons is mediated by cAMP but not PKA.

Relevance to stress-related psychiatric disorders

The noradrenergic system of the LC has long been implicated in the pathophysiology of mood and anxiety disorders (Charney et al., 1995; Wong et al., 2000). Increased CSF levels of CRH have been reported in several studies of patients with major depression, post-traumatic stress disorder, obsessive compulsive disorder, and Tourette's syndrome (Arborelius et al., 1999). Furthermore, increased levels of CRH within the LC of depressed patients have recently been reported (Bissette et al., 2003), and chronic treatment with various antidepressants alters the interaction between CRH and LC neurons (Curtis and Valentino, 1994). Therefore, the direct link between the CRH and noradrenergic systems reported here further supports the notion that the interaction between these transmitters systems in the LC plays an important role in the pathophysiology of mood and anxiety disorders and may be a potential therapeutic target for their treatment.

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