Bradycardic effects of microinjections of urocortin3 into the nucleus ambiguus of the rat

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Short title: Urocortin3 and the nucleus ambiguus

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ABSTRACT

The presence of UCN3 and CRF2Rs has been demonstrated in brain tissue. Nucleus ambiguus (nAmb) is the predominant brain area providing parasympathetic innervation to the heart. Based on these reports, it was hypothesized that activation of CRF2Rs in the nAmb may elicit cardiac effects. Experiments were carried out in urethane-anesthetized, artificially ventilated, adult male Wistar rats. Microinjections of L-glutamate (L-GLU, 5 mM) were used to identify the nAmb. Different concentrations of UCN3 (0.031, 0.062, 0.125, 0.25 and 0.5 mM) microinjected into the nAmb elicited decreases in heart rate (HR) (5.3 ± 1, 22 ± 3.3, 38 ± 4.9, 45.7 ± 2.7 and 27.3 ± 2.3 bpm, respectively). The volume of all microinjections was 30 nl. Blood pressure changes concomitant with decreases in HR were not observed. Bradycardia elicited by microinjections of UCN3 (0.25 mM; maximally effective concentration) into the nAmb was significantly (p < 0.05) attenuated by microinjections of selective CRF2R antagonists (K41498, 0.5 mM and astressin 2B, 0.25 mM) at the same site. Bilateral vagotomy abolished the bradycardic responses to UCN3. These results indicated that activation of CRF2Rs in the nAmb by UCN3 elicited bradycardia which was vagally mediated. UCNs have been reported to exert cardioprotective effects in heart failure and ischemia/reperfusion injury. In this situation centrally induced bradycardia by UCN3 would be beneficial. The results of the present investigation provide a platform for future studies on the role of CRF2Rs in the nAmb in pathological states such as heart failure.
Keywords

bradycardia; heart rate; CRF receptors; CRF2R antagonists, microinjection, vagotomy.
INTRODUCTION

The urocortins belong to the corticotropin-releasing factor (CRF) family of peptides and play important physiological roles in several tissues (32). Included in this family are three peptides: urocortin1 (UCN1), urocortin2 (UCN2) and urocortin3 (UCN3). UCN1 (formerly called urocortin) mediates its actions via both CRF1 and CRF2 receptors and has been reported to increase anxiogenic behavior and adrenocorticotropic release (25, 35). UCN2 (also known as stresscopin-related peptide) has been implicated in central regulation of appetite and autonomic functions and mediates its actions via CRF2 receptors (CRF2Rs) (39). UCN3 consists of 38 amino acids and is structurally closely related to stresscopin which is a 40-amino acid peptide (17, 20). The presence of UCN3 has been demonstrated in brain tissue by in-situ hybridization and immunohistochemistry (21). UCN3 has been implicated in several physiological functions including the recovery phase of stress (18).

The presence of CRFR receptors in the brain has been reported in the literature (13, 45). Urocortins are expected to exert central effects including cardiovascular function because they are transported into the central nervous system (CNS) via a special mechanism (33). Central cardiovascular actions of urocortins are diverse. For example, microinjections of UCN3 into the hypothalamic paraventricular nucleus (PVN) has been reported to increase blood pressure (BP), heart rate (HR) and sympathetic nerve activity (SNA) in anesthetized rats (22) while microinjections of urocortins into the medial subnucleus of the nucleus tractus solitarius (mNTS) elicit decreases in BP, HR and SNA (27, 28, 47). Activation of CRF1Rs in the nucleus ambiguus (nAmb), which is the predominant source of parasympathetic innervation to the heart (4, 8, 24, 25, 30, 31, 44), has been reported to elicit bradycardia (11). This information in the literature prompted our hypothesis that activation of CRF2Rs in the nAmb may elicit cardiac effects.
Accordingly, cardiac effects of direct microinjections of UCN3 into the nAmb were studied in this investigation. Because UCN3 has been identified recently (17, 20), little information is available regarding its central cardiovascular actions.
METHODS

General procedures

Adult male Wistar rats (Charles River Laboratories, Wilmington, MA), weighing 300–380 g, were used in this study (n = 93). The rats were housed in the Comparative Medicine Resources facilities of this institution under controlled conditions with a 12:12-h light-dark cycle. Food and water were allowed ad libitum to the animals. The experiments were carried out with the approval of the Institutional Animal Care and Use Committee of this university and according to the NIH guide for “The Care and Use of Laboratory Animals, 7th Edition, 1996”.

The procedures used in this study have been described in detail in our previous publication and are mentioned here in brief (12). Inhalation of isoflurane (2-3% in 100% oxygen) was used initially to anesthetize the rats. The trachea was cannulated and the rats were artificially ventilated using a rodent respirator (model 683, Harvard Apparatus, Holliston, MA, USA). Then, urethane (1.2-1.4 g/kg) was injected intravenously via a cannula placed in a femoral vein; the concentration of urethane was 0.9 g/ml and total volume of the anesthetic solution (0.4-0.6 ml) was injected in 8-12 aliquots, at 2 min intervals, over a period of 10-12 min. Inhalation of isoflurane was stopped as soon as urethane administration was completed. Adequate depth of anesthesia was confirmed by the absence of a BP response and/or withdrawal of the limb in response to pinching of a hind paw. A cannula was placed in one of the femoral arteries and BP waves were recorded using 1401 A/D converter and Spike 2 software (Cambridge Electronic Design (CED), Cambridge, UK). Mean arterial pressure (MAP) and HR were derived electronically from the BP waves using the same CED data acquisition system. The tidal volume and frequency were adjusted on the ventilator to maintain the end tidal CO₂ at 3.5-4.5%. The rectal temperature was maintained at 37 ± 0.5°C using a rectal probe (RET-1) connected to
TCAT-2A temperature controller (Physitemp Instruments, Clifton, NJ, USA). All of the tracings were stored on a computer hard drive. At the end of the experiment, the rats were deeply anesthetized with a high dose of urethane (1.8-2 g/kg, i.v.), a pneumothorax was produced by an incision in one of the intercostal muscles and cessation of heart beat, which was recorded on line, indicated that euthanasia was complete.

*Bilateral vagotomy*

In these experiments, both the vagus nerves were identified and loose silk threads were placed around them. The rats were then fixed in a stereotaxic instrument and L-GLU (5 mM) and UCN3 (0.25 mM) were microinjected into the nAmb. Forty min later, one of the vagus nerves was sectioned by carefully pulling on the silk loop. After a gap of 3-5 min, second vagus nerve was sectioned in the similar manner. A stabilization period of 15-20 min was allowed after bilateral vagotomy. Microinjections of L-GLU and UCN3 into the nAmb were then repeated and changes in HR responses before and after the bilateral vagotomy were noted.

*Microinjection technique*

We have described this technique in detail in one of our earlier publications (12). The rats were fixed in a stereotaxic instrument, in a prone position, with bite bar 18 mm below the interaural line. Multi-barreled glass-micropipettes (tip size 20-40 μm) were used for microinjections into the nAmb using a dorsal approach. The coordinates for the nAmb were: 0.12 caudal to 0.64 mm rostral to obex, 1.8-2 mm lateral to the midline and 2-2.4 mm below the dorsal surface of the medulla. Controls for microinjections consisted of artificial cerebrospinal fluid (aCSF, pH 7.4). The volume of all microinjections into the nAmb was 30 nl unless indicated otherwise.
Intravenous administration of UCN3

One of the femoral veins of these rats was cannulated and the concentration of UCN3 (0.25 mM, 30 nl) that elicited maximum responses when microinjected into the nAmb was administered intravenously.

Immunohistochemistry

Surgical procedures for immunohistochemical studies were done under aseptic conditions. The rats, anesthetized with pentobarbital sodium (50 mg/kg, i.p.), were placed prone in a stereotaxic assembly. Unilateral microinjections of colchicine (120 μg, 10 μl) were made into the lateral ventricle (coordinates for the lateral ventricle: 0.8-0.9 mm caudal to the bregma, 1.7–1.8 mm lateral to the midline, and 3.8–4.0 mm deep from the dura). Subcutaneous injections of an antibiotic (cefazolin, 30 mg/kg) and an analgesic (buprenorphine, 0.05 mg/kg) were administered twice a day for 2 days. At this time, the rats were deeply anesthetized with urethane (1.8-2 g/kg, i.v.), and heparinized normal saline, followed by 2% paraformaldehyde solution containing 0.2% picric acid, were perfused into the rats. The brains were then dissected out and placed in 2% paraformaldehyde containing 0.2% picric acid for 48 hrs. When the fixation procedure was completed, serial sections of the medulla were cut (40 μm) in a vibratome (1000 Plus Sectioning System, The Vibratome Company, St. Louis, MO, USA). The sections containing the nAmb were used for immunostaining for CRF2R and UCN3. The sections were rinsed for 10 min each with 0.1 M phosphate buffered saline (PBS). The endogenous peroxidase was blocked by 3% hydrogen peroxide in 0.1M PBS (10 min) and the sections were then washed 4 times in 0.1 M PBS, 10 min each.

For CRF2R immunostaining, the sections were incubated with 10% normal rabbit serum (NRS) in 0.1 M PBS containing 0.3% Triton-X (TPBS) for 60 min at room temperature to reduce
nonspecific binding of antibodies. The sections were then rinsed for 10 min with 0.1 M PBS and incubated overnight at 4°C with the primary antibody for CRF2R (host goat; 1:100; sc-20550, Santa Cruz Biotechnology, California, USA; diluted with TPBS containing 3% NRS). The sections were again rinsed with PBS, incubated for 2 hours at room temperature with the secondary antibody (Cy3-rabbit anti-goat IgG; 1:200, #305-165-003, Amax = 550 nm, Emax = 570 nm, Jackson Immuno-Research Laboratories Inc., West Grove, PA, USA; diluted with TPBS containing 3% NRS).

For UCN3-immunoreactivity, the sections were incubated with 10% normal goat serum (NGS) in TPBS for 60 min at room temperature to reduce nonspecific binding of antibodies. The sections were then incubated overnight at 4°C with the primary antibody for UCN3 (host rabbit; 1:500; H-019-29, Phoenix Pharmaceuticals, Inc., Burlingame California, USA; diluted with TPBS containing 3% NGS). The sections were again rinsed with PBS, incubated for 2 hours at room temperature with the secondary antibody (Cy3-goat anti-rabbit IgG; 1:200, #111-165-003, Amax = 550 nm, Emax = 570 nm, Jackson Immuno-Research Laboratories Inc., West Grove, PA, USA; diluted with TPBS containing 3% NGS).

In each experiment, the sections were rinsed in PBS after the incubation with the primary and secondary antibodies was completed. The sections were placed on subbed slides, mounted with Citifluor medium (Ted Pella Inc., Redding, CA, USA) and coverslipped. Laser scanning confocal microscopy was done to capture the images of the sections, 1 μm apart, using an AIR confocal microscope (Nikon Instruments Inc., Melville, NY, USA).
Histological identification of microinjection sites

Microinjection sites were marked by microinjections of diluted (1: 50) green retrobeads (Lumafluor Corp., Durham, NC, USA). The animals were perfused and fixed with 2% paraformaldehyde, serial sections of the medulla were cut (40 µm), mounted on slides, covered with Citifluor Mountant media and coverslipped. The microinjection sites were identified, using a fluorescence microscope, photographed and compared with a standard atlas (34).

Drugs and chemicals

The following drugs and chemicals were used. Astressin 2B, colchicine, D-AP7 (D(-)-2-amino-7-phosphono-heptanoic acid; N-methyl-D-aspartic acid [NMDA] receptor antagonist), NBQX disodium (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo-[f]quinoxaline-7-sulfonamide disodium; non-NMDA receptor antagonist), isoflurane, gabazine (SR95531), K41498, L-glutamate monosodium (L-GLU), strychnine hydrochloride, urethane and UCN3. All of the solutions for the microinjections were freshly prepared in aCSF. The composition of aCSF (pH 7.4) was as follows; NaCl (128 mM), KCl (3 mM), CaCl₂ (1.2 mM), MgCl₂ (0.8 mM), dextrose (3.4 mM) and HEPES (5 mM). Where applicable, the concentration of drugs refers to their salts. The vendors for different drugs and chemicals were as follows: astressin 2B, D-AP7 and NBQX (R & D Systems, Inc., Minneapolis, MN, USA), isoflurane (Baxter Pharmaceutical Products, Deerfield, IL, USA) and UCN3 (American Peptide, Sunnyvale, CA, USA). All other drugs and chemicals were obtained from Sigma Chemicals (St. Louis, MO, USA).
**Statistical analyses**

Maximum changes in HR in response to microinjections of different drugs were expressed as means and standard error of the means (SEM). One-way analysis of variance (ANOVA) followed by Tukey-Kramer’s multiple comparison test was used for concentration-response study and determination of tachyphylaxis. Student’s paired \( t \)-test was used for comparison of the decreases in HR before and after microinjections of L-GLU and UCN3 into the nAmb. The differences were considered to be significant at \( P < 0.05 \).
RESULTS

Concentration-response of UCN3 into the nAmb

In this and other series of experiments, nAmb was always identified by unilateral microinjections of L-GLU (5 mM) which elicited bradycardia (83.6 ± 6, bpm) but no change in BP. Microinjections of different concentrations (0.031, 0.062, 0.125, 0.25 and 0.5 mM) of UCN3 into the nAmb elicited decreases in HR (5.3 ± 1, 22 ± 3.3, 38 ± 4.9, 45.7 ± 2.7 and 27.3 ± 2.3 bpm; Fig.1). The maximal bradycardic responses were elicited by 0.25 mM concentration; therefore, this concentration of UCN3 was selected for further studies in other groups of rats. The onset, peak and duration of the bradycardic responses elicited by 0.25 mM concentration of UCN3 were 5-15 sec, 40-90 sec and 6-8 min, respectively (n = 15). In this and other experiments reported in this study, microinjections (30 nl) of aCSF did not elicit any cardiovascular responses.

Effect of intravenous administration of UCN3 on BP and HR

As mentioned earlier, microinjection of UCN3 (0.25 mM, 30 nl) into the nAmb elicited maximum bradycardic responses. Same concentration of UCN3 when injected intravenously failed to elicit a response.

Reproducibility of UCN3 responses

Three consecutive microinjections of UCN3 (0.25 mM) into the nAmb, at 40 min intervals, elicited similar decreases in HR (45.8 ± 1, 40.5 ± 3.4 and 44.3 ± 1.4 bpm, respectively; P > 0.05; n = 5) indicating that repeated microinjections of UCN3 into the nAmb did not exhibit tachyphylaxis when the interval between microinjections was at least 40 min.
Site specificity of UCN3 responses

Microinjection of UCN3 (0.25 mM) into the adjacent regions of nAmb did not elicit any changes in BP and HR (n = 5). For example, microinjection of UCN3 into a site 0.48 - 0.64 mm rostral, 2.6 – 2.8 mm lateral to calamus scriptorius and 2.5 to 2.8 mm deep from dorsal medullary surface did not show any changes in BP and HR; in this experiment, the baseline values for BP and HR were 95.6 ± 3.4 (mmHg) and 410.2 ± 4.5 (bpm), respectively, which did not change after the microinjection of UCN3 (0.25 mM).

Effect of vagotomy on UCN3-induced bradycardia

Bilateral vagotomy abolished the bradycardic responses elicited by microinjections of L-GLU as well UCN3 into the nAmb (Fig. 2). In this group of rats (n = 5), the decreases in HR induced by microinjections of L-GLU (5 mM) into the nAmb before and after the vagotomy were 80 ± 7.3 and 2.5 ± 1.1 bpm, respectively (P < 0.001). Similarly, the decreases in HR elicited by microinjections of UCN3 (0.25 mM) into the nAmb before and after the vagotomy were 46.7 ± 3.3, and 1.7 ± 1 bpm, respectively (P < 0.001).

Blockade of UCN3-induced responses by selective CRF2 receptor antagonists

The effect of different concentrations (0.125, 0.25, 0.5 and 1 mM) of K41498 (a selective CRF2 receptor antagonist) (19) on UCN3-induced decreases in HR were studied in different groups of rats (n = 24; Table 1). Microinjections of UCN3 (0.25 mM) into the nAmb elicited decreases in HR. After an interval of 40 min, K41498 was microinjected into the nAmb which was followed within 2 min by a microinjection of UCN3 at the same site. Blockade of CRF2Rs by K41498 in the nAmb significantly attenuated the bradycardic responses elicited by microinjections of
UCN3 into the nAmb (Table 1). A typical tracing of the blockade of UCN3 responses by microinjections of K41498 is shown in Fig. 3.

The effect of different concentrations (0.125, 0.25, and 0.5 mM) of another selective CRF2R antagonist (astressin 2B) (40) on UCN3-induced decreases in HR was studied in different groups of rats (n = 18; Table 1). Microinjections of UCN3 (0.25 mM) into the nAmb elicited decreases in HR. Forty min later, astressin 2B was microinjected into the nAmb which was followed within 2 min by a microinjection of UCN3 at the same site. Blockade of CRF2Rs by astressin 2B in the nAmb significantly attenuated the bradycardic responses elicited by microinjections of UCN3 into the nAmb (Table 1). A typical tracing of the blockade of UCN3 responses by microinjections of astressin 2B is shown in Fig. 4. The responses to L-GLU were not altered by either one of the CRF2R antagonists (K41498 or astressin 2B).

The effects of CRF2R antagonists were observed for at least 60 min. Recovery of the UCN3-induced bradycardia after the microinjection of either one of the CRF2R antagonists (K41498 or astressin 2B) at all the concentrations used was not complete within 60 min.

Microinjections of K41498 (0.5 mM; maximally effective concentration) alone into the nAmb did not elicit any significant changes in HR. The values of HR before and after the microinjections of K41498 were 412.5 ± 18.2 and 403 ± 17.4 (bpm), respectively (P > 0.05). Similarly, microinjections of another CRF2R antagonist (Astressin 2B; 0.25 mM) into the nAmb did not elicit any changes in HR; the values of HR before and after the microinjections of this antagonist were 401 ± 9.4 and 396.5 ± 9.1 (bpm) respectively (P > 0.05).
Effect of gabazine and strychnine on UCN3-induced bradycardia

The nAmb was identified as usual using microinjections of L-GLU (5 mM). Five min later, UCN3 (0.25 mM) was microinjected into the nAmb which elicited a decrease in HR (6.4 ± 0.6%). After an interval of 40 min, gabazine (0.01 mM) was microinjected into the nAmb; a decrease in baseline HR (16.3 ± 3.1 bpm) was elicited (n = 5). Two min later, UCN3 (0.25 mM) was again microinjected at the same site; the bradycardic responses (3.6 ± 0.4%) were attenuated significantly (P < 0.001) (Fig. 5A). In another group of rats (n = 5), L-GLU was microinjected to identify nAmb. At the same site microinjection of UCN3 (0.25 mM) elicited bradycardia (6.8 ± 1.2%). After 40 min, strychnine (0.5 mM) was microinjected at the same site; there was no significant change in baseline HR values. Two min later, microinjection of UCN3 (0.25 mM) elicited decrease in HR but this decrease was significantly attenuated (3.7 ± 1.1%; P < 0.0001) (Fig. 5B). The concentrations of gabazine (0.01 mM) and strychnine (0.5 mM) used in this experiment were selected from published literature (10, 23).

Effect of blockade of ionotropic glutamate receptors (iGLURs) on UCN3-induced bradycardia

Identification of the nAmb was done by microinjections of L-GLU (n = 5). Five min later, UCN3 (0.25 mM) was microinjected at the same site and a decrease in HR (8.2 ± 0.7%) was elicited. After an interval of 40 min iGLUR antagonists (D-AP7, 5 mM) and NBQX, 2 mM) were microinjected into the nAmb; a small decrease in baseline HR (11.4 ± 1.7 bpm) was elicited. Within 2 min, UCN3 (0.25 mM) was microinjected again at the same site; UCN3-induced bradycardia (1.5 ± 0.2%) was significantly (P < 0.0001) attenuated. (Fig. 5C). The concentrations of D-AP7 and NBQX used in this experiment were selected from our previous reports (11, 27).
Identification of immunoreactive cells containing CRF2Rs and UCN3

Immunohistochemical procedures (n = 6) showed the presence of cells containing CRF2Rs (Fig. 6A) and UCN3 (Fig. 6D) in the nAmb. Specificity of the primary antibody for CRF2Rs was indicated by lack of staining for CRF2Rs when the primary antibody was either omitted (Fig. 6B) or pre-absorbed by incubation with CRF2R control peptide (Fig. 6C). Similarly, specificity of the primary antibody for UCN3 was indicated by lack of staining for this peptide when the primary antibody was omitted (Fig. 6E) or pre-absorbed by incubation with UCN3 control peptide (Fig. 6F).

Histological identification of microinjection sites

The microinjection sites of L-GLU (5 mM) and UCN3 (0.25 mM) in the nAmb were marked with a microinjection (30 nl) of green retrobeads (Lumafluor Corp) (n = 30) (Figs. 7).
DISCUSSION

The present study showed that microinjections of UCN3 into the nAmb elicited bradycardia which was blocked by prior microinjections of selective CRF2R antagonists (K41498 and astressin 2B) (19, 40). The effects of K41498 and astressin 2B persisted for at least 60 min indicating that the blocking effect of these CRF2R antagonists was long-lasting. This observation suggested that the bradycardic responses were mediated via CRF2Rs in the nAmb. In this context, it may be noted that UCN3 has been reported to act only through CRF2Rs and it is considered to be the endogenous ligand for these receptors (15, 17, 21). There are very few studies in which the presence of CRF2Rs and UCN3 has been determined in the brain tissue. In one study, CRF2R mRNA expression was not detected in the nAmb (45). In another report, nAmb was not mentioned as one of sites containing UCN3 immunoreactivity (21). Contrary to these reports, we observed scattered cells containing CRF2Rs and UNC3 in the nAmb in the present study; availability of good antibodies for CRF2Rs and UCN3 may have facilitated our immunohistochemical studies. Moreover, we have pharmacologically demonstrated the presence of CRF2Rs in the nAmb (7, present study). At the present time, it is not clear under what physiological or pathological conditions endogenous UCN3 is released in the nAmb.

We have previously reported that microinjections of UCN1 into the nAmb elicited bradycardia which was mediated via CRF1Rs (11). The presence of CRF1Rs in the nAmb has been reported in the literature (45). UCN1 acts on both CRF1Rs and CRF2Rs (15). However, it is unlikely that bradycardic responses to UCN1 in the nAmb were mediated via CRF2Rs because these responses were completely blocked by prior microinjections of a selective CRF1R antagonist (11). The bradycardic responses, including their onset, peak and duration, elicited by UCN1 (11) and UCN3 (this paper) in the nAmb were comparable. No significant differences
were observed between the bradycardic responses, including their onset, peak and duration, elicited by UCN1 (11) and UCN3 (this paper) in the nAmb. For example, decreases in HR elicited by maximally effective concentration of UCN1 and UCN3 (0.25 mM) were 46.9 ± 1.7 and 45.7 ± 2.7 bpm, respectively (P > 0.05). Similarly, the onset, peak and duration of the responses elicited by UCN1 and UCN3 (0.25 mM each) were comparable; the onset, peak and duration of the UCN1-induced responses were 5-20 sec, 40-80 sec and 4-10 min, respectively (unpublished observations), and those of UCN3-induced responses were 5-15 sec, 40-90 sec and 6-8 min, respectively (this paper). This observation is consistent with previous reports in which microinjections of UCN1 and UCN3 into the NTS elicited similar cardiovascular responses in urethane anaesthetized rats (27, 28, 47).

CRF2Rs, like CRF1Rs, are coupled to the guanosine triphosphate-binding protein Gs and stimulate adenylate cyclase activity in most systems (1, 15, 51). Although this intracellular signaling pathway mediates most of the actions of CRF2R activation, other signaling pathways may also be involved (16). Activation of CRF2Rs by UCN3 and CRF1Rs by UCN1 may stimulate cardiovagal neurons in the nAmb by an identical mechanism and elicit similar bradycardic responses.

Concentration-response studies using microinjections of UCN3 into the nAmb showed a non-linear bell-shaped response. Several enzymes, peptides, and hormones show similar concentration-responses. This type of concentration-response has been explained by homotropic allostery; in this model, higher concentrations of the peptide bind to a modulator site other than the primary binding site, modifying the function of the receptor, which results in attenuated responses (5).

Microinjections of aCSF into the nAmb did not elicit a response indicating that local
distortion of brain tissue was not responsible for the bradycardic effects elicited by microinjections of UCN3 at the same site. Leakage of UCN3, if any, from the microinjection site into the peripheral circulation was excluded because concentrations of UCN3 microinjections (0.25 mM, 30 nl) that elicited maximum decreases in HR did not elicit a response when injected intravenously. The site-specificity of UCN3-induced bradycardic responses was indicated by the observation that identical microinjections of UCN3 into areas adjacent to the nAmb elicited no responses. Based on our experiments on un-anesthetized decerebrate rats, we have previously reported that urethane anesthesia did not alter the responses to UCN3 qualitatively or quantitatively (28).

Because microinjections of the CRF2R-antagonists alone into the nAmb did not elicit significant changes in basal HR, it was concluded that under normal physiological conditions CRF2Rs in the nAmb were not under tonic control of endogenous UCN3. We have previously reported that UCNs may not be the neurotransmitters normally involved in mediating the cardiac responses to baroreflex activation (11). In this context, it may be noted that there is a general consensus that glutamate is the predominant neurotransmitter in the nAmb mediating reflex bradycardic responses (48).

Based on our current knowledge regarding medullary control of cardiac function (14, 25, 41, 43, 48), the mechanism of the bradycardic responses elicited by UCN3 microinjections into the nAmb can be explained as follows. It is well established that cardiac vagal neurons in the nAmb are generally silent and depend on synaptic inputs for their activity (48). Major inputs to the cardiac vagal neurons in the nAmb include glutamatergic excitatory inputs and GABAergic and glycinerergic inhibitory inputs. The glutamatergic inputs may arise from the NTS; in patch clamp studies electrical stimulation of the NTS activated glutamatergic currents in the cardiac vagal
neurons in the nAmb via NMDA, AMPA and kainate receptors (29). This NTS-nAmb pathway may play a role in baroreflex-induced vagally-mediated reflex bradycardia (48). GABAergic inhibitory input also may arise from the NTS; stimulation of the NTS evokes GABAergic synaptic currents in the cardiac vagal neurons in the nAmb (48, 49). This GABAergic pathway from the NTS to the nAmb may mediate inhibition of cardiac vagal neurons during inspiration. Microinjections of glycine into the nAmb have been reported to increase HR indicating the presence of glycine receptors in this nucleus (9). Anatomical studies have shown that cardiac vagal neurons are innervated by glycine-immunoreactive fibers (3) and receive glycinergic input (46, 50). The bradycardic responses elicited by microinjections of UCN3 were attenuated by microinjections of gabazine and strychnine indicating that UCN3 inhibited GABAergic and glycinergic inputs to the cardiac vagal neurons allowing predomination of the excitatory glutamatergic inputs to these neurons in the nAmb. Excitation of the cardiac vagal neurons in the nAmb by glutamatergic inputs is expected to increase vagal input to the heart and elicit bradycardia. This conclusion is supported by our observation that blockade of iGLURs in the nAmb by microinjections of D-AP7 and NBQX significantly attenuated the bradycardic responses elicited by UCN3. Excitation of neurons in the region surrounding the compact formation of the nAmb by UCN3 via CRF2Rs may have increased the activity of vagal input to the heart causing bradycardia. In this context, it may be noted that direct application of UCNs to neurons in other brain areas of the rat (e.g., cerebellar Purkinje neurons) have been reported to increase their firing rate (6). Recently UCN3 has been reported to produce Ca^{2+} influx and Ca^{2+} release from internal stores in retrogradely labeled cardiovagal neurons in the nAmb; this cellular mechanism may be responsible for activation of these neurons (7).
Perspectives and significance

Stress is one of the common risk factors in cardiovascular diseases. UCNs have been reported to be involved in the regulation of stress, anxiety states, food consumption, and social behaviors (32, 33). It is also known that UCNs may play a role in central cardiovascular regulation (11, 27, 28, 47). UCN3 may be released in the nAmb in yet unidentified pathological states (e.g., heart failure) and elicit bradycardia. This effect would counter-balance tachycardia induced by the disease. UCNs can reach the CNS via a unique transport system (33). Cardioprotective effects of UCNs have been reported in heart failure and ischemia/reperfusion injury (2, 36, 37, 38, 42). These beneficial effects of UCNs may be mediated via direct actions on myocardial tissue. For example, intravenous administration of UCNs elicits beneficial effects in experimental heart failure by increasing cardiac output and reducing peripheral resistance (2, 36, 37, 38, 42). In this situation centrally induced bradycardia by UCN3 would be beneficial. In the present paper we have described the mechanism by which UCN3 exerts bradycardic effects when microinjected into the nAmb. As mentioned in the Introduction section, the importance of nAmb in the central regulation of cardiac function is well established (4, 8, 24, 25, 30, 31, 44). The results of the present study provide a platform on which future studies can be designed for investigating the role of CRF2RS in the nAmb in cardiac regulation during pathological states.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
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LEGENDS FOR FIGURES

Fig. 1. Concentration - response for UCN3 microinjections into the nAmb.
The decreases in HR in response to microinjections of different concentrations of UCN3 into the nAmb elicited bradycardic responses. Maximum bradycardic responses were elicited by microinjections of 0.25 mM of UCN3. The volume of all microinjections was 30 nl. * P < 0.01; ** P < 0.001.

Fig. 2. Bar diagram showing the effect of bilateral vagotomy on L-GLU and UCN3 responses.
Bilateral vagotomy (Vag-X) significantly (**P < 0.001) attenuated the bradycardic responses elicited by microinjections of L-GLU and UCN3 into the nAmb.

Fig. 3. Tracing showing blockade of UCN3 responses in the nAmb by K41498.
A: Bradycardic responses to microinjection of L-GLU (5 mM). B: After allowing an interval of 5 min, the vehicle (aCSF) was microinjected at the same site; no significant changes were observed. C: UCN3 (0.25 mM) was microinjected at the same site; bradycardic responses were elicited. D: After an interval of 40 min to avoid tachyphylaxis to UCN3 responses, K41498 (a CRF2R antagonist; 0.5 mM) was microinjected at the same site; the antagonist elicited no significant responses. E: A two-min interval was allowed for the diffusion of the antagonist into the nAmb and UCN3 (0.25 mM) was again microinjected at the same site; the responses to UCN3 were blocked. F: The responses to L-GLU (5 mM) were unaltered by K41498. PAP: pulsatile arterial pressure; MAP: mean arterial pressure; HR: heart rate.
Fig. 4. Tracing showing blockade of UCN3 responses in the nAmb by astressin 2B.

A: Bradycardic responses to microinjection of L-GLU (5 mM). B: After an interval of 5 min, the vehicle (aCSF) was microinjected at the same site; no significant changes were observed. C: UCN3 (0.25 mM) was microinjected at the same site; bradycardic responses were elicited. D: After an interval of 40 min, astressin 2B (a CRF2R antagonist; 0.25 mM) was microinjected at the same site; the antagonist elicited no significant responses. E: UCN3 (0.25 mM) was again microinjected at the same site; after a 2 min interval; the responses to UCN3 were blocked. F: The responses to L-GLU (5 mM) were unaltered by astressin 2B. PAP: pulsatile arterial pressure; MAP: mean arterial pressure; HR: heart rate.

Fig. 5. The effect of blockade of GABA, glycine and ionotropic glutamate receptors in the nAmb on UCN3 responses. The bradycardic responses elicited by microinjections of UCN3 (0.25 mM) into the nAmb were significantly attenuated by microinjections of gabazine (0.01 mM) (A), strychnine (0.5 mM) (B) and combined microinjections of D-AP7 (5 mM) and NBQX (2 mM) into the nAmb (C). ** P < 0.001. *** P < 0.0001

Fig. 6. Identification of cells immunoreactive for CRF2Rs and UCN3. A: cells immunoreactive for CRF2Rs in the nAmb area (white arrows). B: Control in which the primary antibody for CRF2R was omitted from the protocol. C: Control in which the primary antibody for CRF2R was pre-incubated with CRF2R peptide. D: UCN3-immunoreactive cells in the nAmb (white arrows). E: Control in which the primary antibody for UCN3 was omitted from the protocol. F: Control in which the primary antibody for UCN3 was pre-incubated with CRF2R peptide. Calibration bar in each panel = 50 µm.
Fig. 7. Histological verification of microinjection sites in nAmb. A: A typical nAmb site marked with Lumfluor (30 nl); the site was located 0.24 rostral to the calamus scriptorius, 2.0 mm lateral to the midline, 2.2 mm deep from the medullary surface (arrow). B-H: Drawings of coronal sections at levels 0.64 rostral (B) to 0.12 caudal (H) to the calamus scriptorius showing microinjection sites (dark circles). Each dark circle represents a microinjection site in the nAmb in one rat (n = 30). AP: area postrema; CC: central canal; nAmb: nucleus ambiguus; py: pyramid; 10N: dorsal motor nucleus of vagus; 12N: hypoglossal nucleus
Table 1. Effect of different CRF2R antagonists on UCN3-induced responses

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<thead>
<tr>
<th>n</th>
<th>Antagonist</th>
<th>UCN3-induced decreases in HR (beats/min)</th>
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*P < 0.05; **P < 0.001; ***P < 0.0001.

The antagonists as well as UCN3 (0.25 mM) were microinjected into the nAmb which was always identified by prior microinjections of L-GLU (5 mM).
Fig. 1: Graph showing the decrease in heart rate (bpm) with different concentrations of UCN3 (mM). The concentrations tested are 0.031, 0.062, 0.125, 0.25, and 0.5 mM. The data points are represented with error bars, and significant differences are indicated with asterisks (* for p < 0.05, ** for p < 0.01).
![Graph showing the decrease in HR with L-GLU (5 mM) and UCN3 (0.25 mM). The graph indicates a significant decrease in HR before and after vagal stimulation.](image)

**Fig. 2**
Fig. 5

UCN3-induced % decrease in HR

A: Gabazine (0.01 mM)
B: Strychnine (0.5 mM)
C: D-AP7 & NBQX (5 mM) (2 mM)

Before
After

-10 -5 0

*** **
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