Title: Corticotrophin-Releasing Factor Augments the I_{H} in Rat Hypothalamic Paraventricular Nucleus Parvocellular Neurons \textit{in vitro}

Authors: De-Lai Qiu,\textsuperscript{1,2} Chun-Ping Chu,\textsuperscript{1} Tetsuro Shirasaka,\textsuperscript{3} Hiromasa Tsukino,\textsuperscript{2} Hiroyuki Nakao,\textsuperscript{2} Kazuo Kato,\textsuperscript{1} Takato Kunitake,\textsuperscript{1} Takahiko Katoh,\textsuperscript{2} and Hiroshi Kannan\textsuperscript{1}

Affiliations: Departments of \textsuperscript{1}Physiology, \textsuperscript{2}Public Health, and \textsuperscript{3}Anesthesiology, Miyazaki Medical College, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan

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Corresponding author: Hiroshi Kannan, M.D., Ph.D.

Department of Physiology, Miyazaki Medical College, University of Miyazaki, 5200 Kihara, Kiyotake-cho, Miyazaki-gun, Miyazaki 889-1692, Japan

Phone: +81-985-850870; Fax: +81-985-855805

E-mail: kannanh@med.miyazaki-u.ac.jp
ABSTRACT:

The goal of this study was to characterize the effects of corticotrophin-releasing factor (CRF) on rat paraventricular nucleus (PVN) putative parvocellular neurons using whole-cell patch-clamp recordings and single-cell reverse transcription-multiplex polymerase chain reaction (single-cell RT-mPCR) techniques. Under current-clamp, CRF (10 nM to 600 nM) increased the neuronal basal firing rate and depolarized neurons in a dose-dependent manner. CRF-induced depolarization was unaffected by co-perfusion with tetrodotoxin (TTX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and bicuculline but was completely inhibited by ZD7288. Under voltage-clamp, 300 nM CRF significantly increased the hyperpolarization-activated cation current (I_{H}) in a voltage-dependent manner, shifted the I_{H} conductance-voltage relationship (V_{1/2}) towards depolarization by approximately 7.8 mV, and enhanced the I_{H} kinetics without changing the slope constant (k). Extracellular application of ZD7288 completely blocked I_{H} and the CRF-induced increase in I_{H}. Further, CRF-induced effects were completely blocked by extracellular application of 1 µM α-helical CRF-(9-14) (α-hCRF), a non-selective CRF receptor antagonist, but were not affected by extracellular application of antisauvagine-30, a selective CRF-receptor 2 antagonist. Single-cell RT-mPCR analysis showed that these neurons co-expressed CRF receptor 1 mRNA and CRF receptor 2 mRNA. Furthermore, CRF-sensitive neurons co-expressed HCN1 channel mRNA, HCN2 channel mRNA, and HCN3 channel mRNA but not HCN4 channel mRNA. These results suggest that CRF modulates the subpopulation of PVN parvocellular neuronal function by CRF-receptor 1-mediated potentiation of HCN ion channel activity.
INTRODUCTION

Corticotrophin-releasing factor (CRF) is a 41-amino acid peptide that is synthesized and secreted in many regions of the brain and plays a major role in the coordination of endocrine, autonomic, and behavioral responses to stressful stimuli. CRF is synthesized in the parvocellular neurons of the hypothalamic PVN and is the primary regulator of adrenocorticotropin hormone (ACTH) release from the anterior pituitary in response to stress (Vale et al. 1981; Antoni 1986). When applied in vivo or in vitro, CRF can directly alter neuronal behaviors in several brain regions. For example, CRF excites neurons of the cortex and the forebrain (Eberly et al. 1983), and the excitability of the hippocampus, Purkinje cells, and the dorsal vagal complex is augmented by CRF-mediated reductions in after-hyperpolarization (AHP) (Aldenhoff et al. 1983; Hollrigel et al. 1998; Yamashita et al. 1991; Lewis et al. 2002).

The effects of CRF are mediated by two G protein-coupled receptors, the type-1 and -2 CRF receptors (Chang et al. 1993; Chen et al. 1993; Lovenberg et al. 1995). The expression of both CRF and CRF-receptor 1 (CRFR-1) mRNA in the parvocellular PVN increases in response to various stimuli, including stress (Luo et al. 1994; Makino et al. 1995) and intracerebroventricularly (ICV)-administered CRF (Imaki et al. 1996; Mansi et al. 1996). Other studies have demonstrated that parvocellular PVN neurons showed strong reactivity to an anti-CRFR-1 antibody (Bittencourt and Sawchenco 2000; Chen et al. 2000; Imaki et al. 2001). In contrast, the CRF receptor 2 (CRFR-2)
is expressed in discrete regions, including the lateral septum and the ventromedial hypothalamus in the forebrain and the dorsal raphe and nucleus of the solitary tract in the hindbrain (Chalmers et al. 1995; Van Pett et al. 2000). Furthermore, CRF binds with high affinity to the CRFR-1 but has low affinity for CRFR-2 (Frank et al. 2002).

The autonomous beating of the heart and a considerable number of rhythmic activities in the brain are controlled by the hyperpolarization-activated cation current ($I_{\text{H}}$) (Pape 1996; Lüthi A and McCormick 1999; Robinson and Siegelbaum 2003). $I_{\text{H}}$ channels are stimulated by membrane hyperpolarization, gated by cyclic nucleotides (cAMP, cGMP), and blocked by extracellular Cs$^+$ and ZD7288 (Harris and Constanti 1995; Ludwig et al. 1998; Ghamari-Langroudi and Bourque 2000). Four different isoforms (HCN1-4) of the $I_{\text{H}}$ channel have been cloned (Santoro et al. 1998; Ludwig et al. 1998; Monteggia et al. 2000), and all four isoforms are expressed in rat PVN (Monteggia et al. 2000). Regulation of these HCN channels may occur via cyclic adenosine monophosphate (cAMP), cyclic guanine monophosphate (cGMP), and/or Ca$^{2+}$ (Pape 1996; Ludwig et al. 1998; Biel et al. 2002).

The PVN comprises magnocellular neurons that secrete oxytocin (OT) and vasopressin (VP), neurosecretory parvocellular neurons that secrete hypophysiotropic hormones, and non-neurosecretory, pre-autonomic parvocellular neurons (Swanson and Sawchenko 1983; Liposits 1993). In spite of the fact that the $I_{\text{H}}$ channels and CRF receptors are both present in the rat PVN neurons, the effect of CRF on activities of the HCN channels is unknown. In the present study, we
used the whole cell patch-clamp and single-cell RT mPCR method to examine the effects of CRF on rat PVN putative parvocellular neurons in vitro.

MATERIALS AND METHODS

Hypothalamic slice preparation. Hypothalamic slices were prepared from P12- to P14-day-old male Wistar rats, as previously described (Qiu et al. 2003). All experiments were approved by the Ethics Committee of the Miyazaki Medical College and were conducted in accordance with international guidelines on the ethical use of animals in a laboratory. Briefly, the brain was quickly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) consisting of (in mM) 140 NaCl, 3 KCl, 1.3 MgSO$_4$, 1.4 NaH$_2$PO$_4$, 11 D-glucose, 5.2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 2.4 CaCl$_2$, and 3.25 NaOH. The pH was 7.3, the osmolarity was 290-300 mOsm, and the fluid was aerated with 100% O$_2$. Coronal slices, including the PVN, with a thickness of 250 µm were generated using a vibrating brain slicer (DSK-2000; Dosaka, Kyoto, Japan). The slices were incubated for at least 1 h in a chamber filled with equilibrated ACSF at room temperature (24-26°C) before recordings started.

Electrophysiology. Patch pipettes were made with a puller (PB-7; Narishige, Tokyo, Japan) from thick-wall borosilicate glass (GD-1.5; Narishige). They were filled with a solution consisting of (in mM) 130 potassium gluconate, 10 HEPES, 10 KCl, 1 CaCl$_2$, 5 EGTA, 1 MgCl$_2$, 2 Na$_2$ATP, and 0.5 Na$_3$GTP. The pH was adjusted to 7.2 with KOH. Patch pipette resistances were 5-7 MΩ in the bath, with series of resistance in the range of 10-20 MΩ, compensated by 80%. The liquid junction potential (10 mV) was corrected according to the method described by Neher (1992). Membrane potentials and/or currents were monitored with an Axopatch 200B amplifier (Axon Instruments,
Foster City, CA, USA), filtered at 1-5 kHz, and acquired through a Digidata 1200 series analog-to-digital interface on a personal computer using Clampex 7.0 software (Axon Instruments). Whole-cell recordings were made from microscopically identified cells. Once stable recording conditions were obtained, a PVN neuron was identified electrophysiologically as type I (magnocellular) or type II (parvocellular) according to previously established criteria by current-clamp in standard ACSF: type-I neurons displayed transient outward rectification, while type-II neurons did not (Luther et al. 2000). In order to study the effects of CRF on $I_{\text{H}}$, the PVN neurons were also identified electrophysiologically as inward rectification-expressing neurons or non-inward rectification-expressing neurons according to previously established criteria by current-clamp in standard ACSF (Luther et al. 2000; Qiu et al. 2003). Only inward rectification-expressing putative parvocellular neurons were included in this study. Selected traces were stored on a computer hard drive, and all data were archived on a 4.7 GB DVD-RAM.

Cytoplasm harvest and reverse transcription. Harvesting of cytoplasm and reverse transcription were carried out as previously described (Liss et al. 1999). After whole-cell recording, the cytoplasm was aspirated into the patch pipette by applying gentle negative pressure in the pipette while maintaining a tight seal. The pipette contents (8 µl) were then expelled into a 0.5 ml test tube containing the reagents for reverse transcription. First-strand cDNA was synthesized for 1 h at 42°C. The total volume of the reaction was 20 µl, containing 5 µM of a random hexamer primer, 10 mM of dithiothreitol (DTT), and 200 µM each of deoxyNTP (dNTP), a 5x first-strand buffer (3 µl), 40 U RnaseOUT™ Recombinant Ribonuclease Inhibitor, and 100 U SuperScript™ Rnase H’Reverse
Transcriptase, all purchased from Invitrogen (Life Technologies, Inc.). The single-cell cDNA was kept at –70°C until PCR amplification.

**Multiplex and nested PCR.** PCR amplification was performed with a thermal cycler (Gene Amp PCR system 9700; Perkin-Elmer, Norwalk, CT, USA) using a fraction (4.5 µl) of the single-cell cDNA as a template. The first fraction of cDNA was used to screen for glyceraldehyde-3-phosphate dehydrogenase (GAPDH); the second fraction (4.5 µl) of cDNA was used to screen for CRFR-1 mRNA and CRFR-2 mRNA; and the third fraction (4.5 µl) of cDNA was used to screen for HCN1-4 channel mRNA. The first multiplex-PCR was performed as a hot start in a final volume of 30 µl containing 4.5 µl cDNA, 100 pmol of each primer, and 0.3 mM of each dNTP, a 3 µl 10x PCR buffer, and 3.5 U HotStarTaq DNA Polymerase (Qiagen K.K., Tokyo, Japan) in a Gene Amp PCR system 9700 with the following cycling protocol: (1) 15 min at 95°C; (2) 30 cycles of 1 min at 94°C, 1.5 min at 57°C, and 2 min at 72°C; (3) 10 min at 72°C; and (4) holding at 4°C. Nested-PCR amplifications were carried out with 2.5 µl of the first PCR product in individual reactions using the following modifications: 3.0 U HotStarTaq DNA Polymerase and 0.2 mM dNTP. The second round was performed as follows: (1) 15 min at 95°C; (2) 35 cycles of 45 s at 94°C, 1 min at 56°C, and 1 min at 72°C; (3) 10 min at 72°C; and (4) holding at 4°C.

The nested primer sequences were as follows: GAPDH (accession No. NM_017008) external sense: 5′-GATGGTGAAGGTCGGTGTG (position 849), external antisense:
5'-GGGCTAAGCAGTTGGTGTT (position 1318); GAPDH internal sense: 
5'-TACCAGGGCTGCTTCTCT, internal antisense: 5'-CTCGTGTTCTACACCCCATC (361 bp); 
CRFR-1 (accession No. NM_030999) external sense: 5'-GCCGCTACAATTACTTCA (position 1299), 
internal sense: 5'-GGACTGCTTGATGCTGTGAA (position 1958); CRFR-1 internal sense: 
5'-GTGGATGTTCGTCTGCATTG, internal antisense: 5'-CACAAAGAAGCCCTGAAAGG (394 bp); 
CRFR-2 (accession No. NM_022714) external sense: 5'-TACTGCAACAGACCTTGGA (position 330), 
external antisense: 5'-ACCAGCACTGCTATTCTCA (position 982); CRFR-2 internal sense: 
5'-CCCTAGTGGAGAGCATGC, internal antisense: 5'-AGGTGGTGATGAGGTTTCCAG (303 bp); 
HCN1 (accession No. NM_053375) external sense: 5'-CTGACATGCGCCAGAAGATA (position 1315), 
internal antisense: 5'-GATTGGTGATGAGGTTTCCAG (position 1998); HCN1 internal sense: 
5'-CAACTTCAACTGCGGAAC, internal antisense: 5'-CCTTGGTCAGCCAGCATATT (254 bp); 
HCN2 (accession No. AF247451) external sense: 5'-TCATCGTGGAGAGGGAAATC (position 749), 
external antisense: 5'-GGCATTTGTGGAGGACAT (position 1310); HCN2 internal sense: 
5'-ACTACGCACTCGTGCTTTTC, internal antisense: 5'-CGTGCCAATGAACATAGC (419 bp); 
HCN3 (accession No. NM_053685) external sense: 5'-TCGGACACTTTCTTCTGCT (position 394), 
external antisense: 5'-TGACTCATGGCCTTGAACAG (position 920); HCN3 internal sense:
5′-TTCTGGTGACCTGATTTC, internal antisense: 5′-CACAGCAGCAACATCATTCC (269 bp); HCN4 (accession No. NM_021658) external sense: 5′-ATCGTGGTGAGGACAACA (position 1179), external antisense: 5′-CCGATGAACATGGCATAGC (position 1759); HCN4 internal sense: 5′-GGAGACTCGCATTGACTCG, internal antisense: 5′-AGCCAGACGTACAGACATGC (405 bp). To investigate the presence and size of the amplified fragments, 10 µl aliquots of PCR products were separated by electrophoresis in an agarose gel (2%) and visualized by ethidium bromide staining. All individual PCR products were verified several times by direct sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit and the Applied Biosystems PRISM 310 Genetic Analyzer (ABI, Foster City, CA, USA). A sequence comparison was performed using the BLAST database.

RNA isolation and cDNA preparation for control reactions. Poly(A)+ RNA was prepared from fresh hypothalamus tissue of 13-day-old Wistar rats using the Micro-to-Midi Total RNA Purification System (Invitrogen). Reverse transcription was performed with 250 µg of the poly(A)+ RNA, as described above. The RNA was diluted and used as a positive (+ reverse transcriptase [+RT]) or negative (-RT) control for the PCRs. All nine PCR fragments were detected routinely in the positive control when using the PCR protocol described above. The negative controls of single cells were carried out in parallel with single-cell experiments, excluding only the harvesting procedure, resulting in no detectable bands (n = 10).
Chemicals. Reagents included human/rat CRF (Peptide Institute, Inc., Japan), α-helical CRF-(9-14) (Sigma-Aldrich, St. Louis, MO, USA), anti-sauvagine (11-40) (Bachem AG, Bubendorf, Switzerland), ZD7288 (Tocris Cookson, Inc., Ballwin, MO, USA), tetrodotoxin (TTX, Sigma-Aldrich, St. Louis, MO, USA), 6-cyano-7-nitroquinoxaline-2 3-dione (CNQX), bicuculline, and CsCl (Sigma, Ballwin, MO). ZD7288 was prepared as a 50 mM stock solution (in H$_2$O) and stored at −20°C until use. All other drugs were dissolved in ACSF. In voltage-clamp, tetrodotoxin (TTX, 0.5 µM) and BaCl$_2$ (100 µM) were included in the external recording solutions to block the voltage-gated Na$^+$ channels and the Ba$^{2+}$-sensitive K$^+$ current and express the I$_{\text{h}}$ current (Cardenas et al. 1999).

Data analysis. Data were analyzed using Clampfit 8.0 (Axon Instruments) and are expressed as mean ± SEM. I$_{\text{h}}$ was determined by subtracting I$_{\text{ins}}$ from I$_{\text{ss}}$ at each hyperpolarizing voltage step using the following equation:

$$I_{\text{h}} = I_{\text{ss}} - I_{\text{ins}}. \quad (1)$$

In addition, I$_{\text{h}}$ conductance ($G_{\text{h}}$) was estimated as the amplitude of I$_{\text{h}}$ measured at various potentials (V) divided by the driving force (V − E$_{\text{h}}$), where E$_{\text{h}}$ is the reversal potential of I$_{\text{h}}$ (Ghamari-Langroudi and Bourque 2000) as follows:

$$G_{\text{h}} = I_{\text{h}}/(V - E_{\text{h}}). \quad (2)$$

The value of E$_{\text{h}}$ was arbitrarily set at -33 mV, which reflects the median E$_{\text{h}}$ reported in our previous study (Qiu et al. 2003).
Differences between mean values recorded under control and test conditions were evaluated using one-way ANOVA with Tukey’s post-hoc test with the SPSS Medical Pack. Differences were considered statistically significant at P < 0.05.

RESULTS

Effects of CRF on the PVN parvocellular neurons in current-clamp

A total of 231 PVN neurons were characterized as type-II neurons under whole-cell current-clamp (Luther et al. 2000; Qiu et al. 2003). Responsive neurons exhibited a lack of transient outward rectification in response to a series of depolarizing current pulses delivered at a hyperpolarized membrane potential (Fig. 1A). Eighty percent (185/231) of type-II neurons displayed time-dependent inward rectification during the hyperpolarizing pulses (Fig. 1, A and B), and this response was blocked by 70 µM ZD7288 (Fig. 1B) or by 3 mM Cs⁺ (not shown). Under voltage-clamp, these neurons exhibited a hyperpolarization-activated ZD7288-sensitive inward current (Fig. 1, C and D). These properties are consistent with hyperpolarization-activated inward current (Iₜₜ) conductance (Ludwig et al. 1998; Santoro et al. 1998; Qiu et al. 2003), indicating that hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels exist in these neurons.

Under current-clamp, applications of CRF in concentrations ranging from 30 nM to 600 nM resulted in depolarization and increased the firing rate in a concentration-dependent manner in CRF-sensitive neurons when the holding potentials were ~60 mV (Fig. 2, A and B). To determine whether the CRF-induced depolarization was due to a direct effect of CRF on the PVN neurons, the amplitude of the CRF-induced membrane depolarization was determined in the absence and
presence of tetrodotoxin (TTX, 0.5 µM), 6-cyano-7-nitroquinoxaline-2-3-dione (CNQX, 10 µM), and bicuculline (10 µM). In eight neurons, CRF (300 nM) induced a 5.96 ± 0.53 mV depolarization that recovered to baseline upon washout. Following 10 min of perfusion with 0.5µM TTX, 10µM CNQX, and 10µM bicuculline, reapplication of 300 nM CRF induced a depolarization of 5.92 ± 0.67 mV, indicating that CRF increased directly with the level of depolarization (Fig. 2, C and D, P > 0.05). The CRF-induced depolarization was in a concentration-dependent manner (Fig. 2E). Further, CRF significantly increased the I_h according to the hyperpolarizing current pulses, resulting in a greater depolarizing ‘sag’ (Fig. 3, B and F, # P < 0.05 vs. control, n = 7), and decreased the amplitude of AHP in CRF-sensitive parvocellular neurons (Fig. 3, A and E, # P < 0.05 vs. control, n = 7). The I_h channel selective blocker, ZD7288, completely prevented a CRF-induced decrease in AHP without significantly affecting the amplitude of AHP in the control condition (Fig. 3, C and E, n = 7). ZD7288 treatment also prevented the ‘sag’ and the effects of CRF on the ‘sag’ (Fig. 3, D and F, n = 7).

**CRF augmented I_h in voltage-clamp**

In the presence of TTX (0.5 µM) and Ba^{2+} (100 µM), application of 300 nM CRF to CRF-sensitive neurons with voltage-clamp at –60 mV produced a negligible inward current (8.2 ± 2.3 pA, n = 10). However, when neurons were held at –50 mV and a series of 1-sec hyperpolarizing voltage steps from –50 mV to –120 mV was applied, CRF induced a significant increment in instantaneous current (I_{ins}) at step potentials less than –90 mV (Fig. 4, A and B, * P<0.05, n = 7) and a steady-state current (I_{ss}) at step potentials less than –60 mV (Fig. 4, A and C, * P<0.05). In addition, the I_h (I_{ss} - I_{ins}) current was increased at step potentials less than –60 mV (Fig. 4D).
Furthermore, we estimated the effect of CRF on $I_{\text{H}}$ conductance ($G_{\text{H}}$) (see METHODS). The mean $G_{\text{H}} - V$ relations are shown in Fig. 4E. Note that CRF enhanced the $I_{\text{H}}$ conductance at step potentials less than $-60 \text{ mV}$ (* $P < 0.05$ vs. control). The modified Boltzmann equation was used as follows:

$$G_{\text{H}(V)} = \frac{1}{1 + e^{(V - V_{1/2})/k}},$$

where $G_{\text{H}(V)}$ is the fraction of maximal $G_{\text{H}}$ observed at $V$, $k$ is the slope factor, and $V_{1/2}$ is the half-maximal voltage. The mean values of the Boltzmann equation were as follows: $V_{1/2} = -93.3 \pm 2.4 \text{ mV}$, $k = 11.5 \pm 1.8$ in the control and $V_{1/2} = -85.5 \pm 2.8 \text{ mV}$, $k = 12.91 \pm 1.8$ during the application of CRF (* $P < 0.05$ vs. control). These data suggest that CRF produced a significant shift in $V_{1/2}$ to a more depolarized potential ($7.8 \pm 1.2 \text{ mV}$) and that the slope factor values were not altered by CRF ($P > 0.05$ vs. control).

The time course of the activation of $I_{\text{H}}$ was obtained by analyzing the rising phase of the CRF-induced $I_{\text{H}}$ current that was evoked by hyperpolarizing steps to various voltages. As shown in Fig. 5A and C, the $I_{\text{H}}$ current traces were fit to a single exponential function of the form $A_t = A_\infty (1 - e^{-t/\tau})$, where $A_t$ is the amplitude of $I_{\text{H}}$ at time $t$, $A$ is the amplitude of $I_{\text{H}}$ at a steady state, and $\tau$ is the activation time constant (Ghamari-Langroudi and Bourque 2000; Qiu et al. 2003). The addition of CRF (300 nM) reversibly enhanced the $I_{\text{H}}$ activation. The CRF-mediated enhancement of $I_{\text{H}}$ was also accompanied by reversible acceleration of the HCN channel kinetics exhibiting decrements of the time constant. As shown in Fig. 5B, when neurons were held at $-50 \text{ mV}$ and a 1-s hyperpolarizing voltage step was held from $-50 \text{ mV}$ to $-120 \text{ mV}$, 300 nM CRF induced a significant decrease of the time constant. The decrement of the time constant appeared approximately 50 s after CRF exposure and peaked at approximately 100 s, with a maximal decrement of $\tau$ from $\sim 200 \text{ ms}$ to $\sim 140 \text{ ms}$ for
approximately 200 s. Figure 5D reveals the plots of the mean activation time constants (n = 7) against the voltage steps. The mean $\tau$ of CRF-sensitive neurons decreased from ~700 ms at –70 mV to ~200 ms at –120 mV and exhibited fast kinetics. CRF enhanced the $I_H$ channels kinetics, exhibiting decrements of $\tau$ at step potentials less than –60 mV (P < 0.05 vs. ACSF).

To confirm whether CRF augmented $I_H$, the effects of CRF on PVN neurons in the presence of the hyperpolarization-activated cyclic-nucleotide-gated channel (HCN)-specific antagonist, ZD7288, were examined. In current-clamp, the application of ZD7288 induced a slight hyperpolarization in CRF-sensitive neurons and prevented CRF-induced depolarization (Fig. 6, A and B, *P < 0.05 vs. CRF, n = 5). In voltage-clamp, CRF increased $I_{SS}$ according to the hyperpolarizing pulses (Fig. 6, C and D, n = 4). ZD7288 blocked $I_{SS}$ and CRF-induced increments of $I_{SS}$, and the I-V relationships became linear in the presence of ZD7288 (Fig. 6, C and E, n = 4). This finding confirmed that CRF treatment resulted in the potentiation of HCN channels activities in PVN CRF-sensitive neurons.

**CRF-augmented $I_H$ is not mediated by CRF receptor 2**

To establish the pharmacological profile of CRF receptors that mediated the CRF-enhanced $I_H$, the CRFR-1 and CRFR-2 non-selective antagonist $\alpha$-helical CRF- (9-14) ($\alpha$-helCRF, 1 µM) and a selective CRFR-2 antagonist, anti-sauvagine-30 (aSvg, 200 nM), were applied to PVN CRF-sensitive neurons. The effects of CRF (300 nM) on $I_{SS}$ and $I_H$ in the absence and presence of $\alpha$-helCRF and aSvg (after perfusion $\alpha$-helCRF or aSvg for 10 min) were examined under voltage-clamp. CRF (300 nM) significantly increased the $I_{SS}$ and $I_H$, and $\alpha$-helCRF completely blocked the CRF-induced augmentation of $I_{SS}$ and $I_H$ (Fig. 7, A, B, and E; n = 6). However, aSvg (200 nM) did not prevent the CRF-induced augmentation of $I_{SS}$ and $I_H$ (Fig. 7, C, D, and F; n = 6).
Even the high concentration of aSv (1 µM) did not prevent the CRF-induced augmentation of \( I_{\text{H}} \) (\( n = 2 \), not shown). These data indicate that CRF-induced increase in \( I_{\text{H}} \) was not mediated by CRFR-2.

**CRF-sensitive neurons expressed CRF receptors mRNA and HCN channels mRNA**

After completion of the electrophysiological recording, the CRF-sensitive neurons (\( n = 20 \)) were screened for GAPDH, CRFR-1, CRFR-2, and HCN1-4 channels mRNA using the single-cell RT-mPCR technique. Screening of rat hypothalamic total RNA (positive control) resulted in the detection of all the specific mRNAs, each corresponding to the size predicted by its mRNA sequence (Fig. 8). The identities of all PCR fragments were verified by direct sequencing. All the neurons (20/20) expressed GAPDH, CRFR-1, CRFR-2, HCN1, HCN2, and HCN3 channel mRNAs but not HCN4 channel mRNA (Fig. 8).

**DISCUSSION**

**CRF excited PVN neurons by enhanced \( I_{\text{H}} \)**

The previous studies demonstrated that CRF has a depolarizing effect on the majority of the central nervous system (CNS) neurons, most likely due to a decrease in the amplitude of after-hyperpolarization (AHP) (Eberly et al. 1983; Yamashita et al. 1991; Hang and Storm 2000; Lewis et al. 2002). However, our data showed that CRF evoked a depolarization and increased the neuronal excitability in PVN CRF-sensitive neurons by enhanced \( I_{\text{H}} \) channels activities but not directly by attenuated \( \text{Ca}^{2+} \)-activated \( K^+ \) channels activities. This is supported by several findings. First, the PVN CRF-sensitive parvocellular neurons displayed a ZD7288-sensitive, time-dependent inward rectification during the hyperpolarizing pulses, and the single-cell RT-PCR result indicated
that these neurons expressed HCN1, HCN2, and HCN3 channels mRNA. The HCN channels were partially active at the holding potential (Yaji and Sumino 1998; Qiu et al. 2003), and CRF enhanced $I_{H}$ at the holding potentials, inducing a depolarization in the CRF-sensitive neurons in current-clamp. Under voltage-clamp, CRF enhanced HCN channels kinetics at membrane potentials below $-50$ mV and produced a significant shift in $V_{1/2}$ to a more depolarized potential. Previous reports demonstrated that $I_{H}$ plays a significant role in setting both the resting membrane potential (RMP) and the baseline level of excitability of hippocampal GABAergic interneurons found in the stratum oriens of area CA1 (Lupica et al. 2001) and that the presence of $I_{H}$ in magnocellular neurosecretory cells of the rat supraoptic nucleus provides an excitatory drive that contributes to phasic and tonic firing (Ghamari-Langroudi and Bourque 2000). Furthermore, ZD7288 completely blocked the CRF-induced depolarization and the increments in $I_{Ins}$ and $I_{SS}$, which reflect an increase in tonically activated $I_{H}$ conductance (Mayer and Westbrook 1983; Yaji and Sumino 1998; Qiu et al. 2003). Moreover, ZD7288 completely abolished the CRF-induced augmentation in $I_{H}$ and prevented a CRF-induced decrease in the amplitude of AHP in current-clamp, which indicated that the enhancement of HCN channels activities induced a decrement of the $Ca^{2+}$-activated $K^+$ channels activities and attenuated the amplitude of the AHP (Galligan et al. 1990; Linden et al. 2003).

**Potential mechanisms of CRF-regulated HCN channels**

CRFR-1 mRNA levels in the PVN increase in response to stress (Luo et al. 1994; Makino et al. 1995) and ICV-administered CRF (Imaki et al. 1996; Mansi et al. 1996). CRFR-2 is also expressed in the PVN of the hypothalamus (Lovenberg et al. 1995). Our single-cell RT-mPCR results showed
that the CRF-sensitive neurons co-expressed CRFR-1 mRNA and CRFR-2 mRNA, indicating that CRFR-1 and CRFR-2 co-existed in the CRF-sensitive neurons. The CRFR-1 and CRFR-2 non-selective antagonist, α-helCRF (De Souza 1987), attenuated the CRF-induced augmentation of I_h and suggested that CRF-mediated effects on PVN neurons were mediated by CRF receptors. However, the selective CRFR-2 antagonist, αSVG (Lawrence et al. 2002), did not attenuate the CRF-induced augmentation of I_h in PVN neurons, suggesting that the CRF-induced increase in excitation and the activities of I_h in PVN neurons were not mediated by CRFR-2. A previous study demonstrated that the CRF binds with high affinity to CRFR-1 and with low affinity to CRFR-2 (Frank et al. 2002). Thus, these data suggest that the augmentation of I_h and the increase of neuronal excitability are mediated via the CRFR-1 rather than via the CRFR-2 receptor.

CRF is a potent activator of adenylate cyclase and cAMP production (Facci et al. 2003). After binding to CRFR-1, CRF couples to the stimulatory G protein (Gs), leading to the stimulation of adenylate cyclase and the activation of protein kinase A (PKA) and other cAMP pathway events, increasing the production of cAMP (Dautzenberg and Hauger 2000, 2002; Grammatopoulos and Chrousos 2002). A key property of neuronal HCN channels is their regulation by neurotransmitters and hormones that act via cAMP, cGMP, or intracellular Ca^{2+} (Pape, 1996); cAMP and cGMP modulate HCN channel activity via direct interaction with the cyclic nucleotide-binding domain protein of the C-terminus (Ludwig et al. 1998). A recent report demonstrated that multiple
neurotransmitter receptor systems coupled both positively and negatively to the cAMP synthesis (via the Gs- and Gi-proteins, respectively) and then steadily up- and down-regulated HCN channels activities (Pape 1996; Frere and Luthi 2004).

**Physiological significance**

CRF is a key neurotransmitter that mediates the endocrine, autonomic, and behavioral responses to a variety of stressors (Vale et al. 1981; Smagin et al. 2001). CRF is synthesized in the parvocellular neurons of the hypothalamic PVN and is the primary regulator of the release of the adrenocorticotropic hormone (ACTH) from the anterior pituitary in response to stress (Vale et al. 1981; Antoni 1986). The PVN contains putative pre-autonomic neurons that directly project to the intermediolateral cell column of the spinal cord (Badoer 2001). The increased-endogenesis CRF may directly modulate the PVN putative pre-autonomic neurons by enhancing HCN channels activities via CRFR-1, thus activating the sympathetic nervous center in the spinal cord, which results in increases in blood pressure, heart rate, and plasma norepinephrine (Chu et al. 2004).

Collectively, we propose that CRF binds to CRFR-1, resulting in increased intracellular cAMP and leading to an increment in HCN channels activity and neuronal excitation. This response may contribute to the activation of autonomic centers in the brain stem and spinal cord that regulate blood pressure, heart rate, and plasma norepinephrine.
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Acknowledgments: This work was partially supported by a Grant-in-Aid for Scientific Research (14370024) from the Ministry of Education, Science, Sports, and Culture, Japan and by the KOHNAN Asia Scholarship Foundation. This study was also performed as a part of the Japanese Center of Excellence Program (Section of Life Science).
Figure 1. Electrophysiological properties of PVN CRF-sensitive neurons. A: The neuron displayed time-dependent inward rectification and lacked transient outward rectification (black arrow) in response to a series of depolarizing current pulses delivered at a hyperpolarized membrane potential. B: The neuron displayed inward rectification (sag), which was blocked by 70 µM ZD7288 in response to a -60 pA hyperpolarizing current pulse. C: Current traces elicited by 1s–120 mV hyperpolarizing voltage steps (Vh = –50 mV) in the absence and presence of 70 µM ZD7288. The neuron displayed I_H, which was blocked by ZD7288 in the voltage-clamp. D: Summary of the hyperpolarizing voltage steps versus the I_H current amplitude in ACSF (○) and in the presence of 70 µM ZD7288 (●), n = 6.

Figure 2. Effects of CRF on PVN CRF-sensitive neurons in current-clamp. A_1, A_2, and A_3 are the responses to 30 nM, 100 nM, and 600 nM CRF, respectively (bar, V_h = –60 mV). B_1, B_2, and B_3 are the instantaneous spike rates of the neurons in A_1, A_2, and A_3, respectively. CRF elicited increases in the firing-action potential in a dose-dependent manner. C, 300 nM CRF (bar) provoked a reversible membrane depolarization accompanied by an increase in the firing rate (upper), and the CRF-induced depolarization was unaffected by pre-perfusion with 0.5 µM TTX. D, Summary of the data illustrating the 300 nM CRF-induced depolarization in the absence and presence of 0.5 µM TTX + CNQX (10 µM) + bicuculine (10 µM) (P > 0.05, n = 8). E, The concentration-response curve for the CRF-induced depolarization. The number of neurons tested for each concentration is indicated near the bars.
Figure 3. CRF enhanced the activation of $I_{hi}$ following reduced AHP in current-clamp. A: Representative traces of AHP evoked in ACSF and in the presence of 300 nM CRF. To evoke single-action potentials, neurons were maintained at -60 mV before passing a short (10 ms) depolarizing current pulse of sufficient intensity to evoke an action potential at the offset of the pulse (upper). B, The neuron displayed inward rectification (sag) in response to a -60 pA hyperpolarizing current pulse in the absence and presence of 300 nM CRF. C, Representative traces of AHP evoked in ZD7288 (70 µM) and ZD7288-co-perfused CRF. D, ZD7288 (70 µM) abolished the ‘sag’ and blocked the CRF (300 nM)-induced effect. E, Summary of the data illustrating the AHP amplitude in the ACSF, CRF, ZD7288 (70 µM) and ZD7288 co-perfused with CRF (# p < 0.05, n = 7). F, Bar graph of the depolarizing ‘sag’ in ACSF, CRF, ZD7288, and ZD7288 co-perfused with CRF (# p < 0.05, n = 7).

Figure 4. Effects of CRF on PVN neurons in voltage-clamp. A, In the presence of BaCl$_2$ (100 µM), the current traces were elicited by a series of 1s hyperpolarizing voltage step CRF-sensitive decrements (10 mV decrements; $V_h$ = -50 mV) in ACSF and during the application of 300 nM CRF. B, Plots of instantaneous current in the control (○) and during the application of 300 nM CRF (●) against the membrane potential (↑ shown in A). C, Plots of the steady-state current in the control (○) and during the application of CRF (●) against the membrane potential (↑ shown in A). D, Plots of the $I_{hi}(I_{SS} - I_{in})$ in the control (○) and during the application of CRF (●) against the membrane potential. E, The current ($I_{hi}$) data shown in D were converted into conductance ($G_{hi}$) using the equation, $G_{hi} = I_{hi}/(V + 33)$ (Qiu et al. 2003; $V$ is the test voltage). The solid lines are the best fit through the data.
points using the Boltzmann equation (ACSF○; 300 nM CRF●; n = 7). The mean values were as follows: $V_{1/2} = -93.3 \pm 2.4$ mV, $k = 11.5 \pm 1.8$ in the control and $V_{1/2} = -85.5 \pm 2.8$ mV, $k = 12.91 \pm 1.8$ during the application of CRF. *$P < 0.05$ vs. ACSF.

Figure 5. Acceleration of $I_H$ activation kinetics by CRF. A, In the presence of TTX (0.5 µM) and BaCl2 (100 µM), the current traces were elicited by hyperpolarizing steps to −120 mV from a holding potential of −50 mV under control conditions, during addition of CRF, and after CRF washout. B, Time constant of the exponential fit ($\tau$) for the same experiment plotted versus time. Each circle shows the $\tau$ value for a single response evoked at 0.05 Hz. Black bar, 300 nM CRF application. C, $I_H$ traces evoked by steps to various voltages in the ACSF and during the application of 300 nM CRF. Superimposed on each trace is a monoexponential fit of the data points (a solid line extending to the right). The time constant used in the fits ($\tau$) is indicated beside each trace. D, Plots of the mean $I_H$ activation time constants (n = 7) against the voltage steps. *$P < 0.05$ vs. ACSF.

Figure 6. ZD7288 blocked the CRF-induced responses. A, CRF (100 nM, bar) provoked a reversible membrane depolarization accompanied by an increase in the firing rate, while 70 µM ZD7288 blocked CRF-induced responses. B, Summary of the data illustrating the neuronal membrane potential in the presence of 100 nM CRF, 70 µM ZD7288, and 100 nM CRF + 70 µM ZD7288 (*$P < 0.05$ vs. 100 nM CRF, n = 5, Vh = -60 mV). C, In the presence of TTX (0.5 µM) and BaCl2 (100 µM), the current traces were elicited by 1s −120 mV hyperpolarizing voltage steps (Vh = −50 mV) under ACSF, 300 nM CRF, 70 µM ZD7288, and 70 µM ZD7288 + 300 nM CRF. D, Plots of
steady-state current in the control (○) and during the application of CRF (●) against the membrane potential (↑ shown in C, n = 4). E, Plots of the steady-state current in the ZD7288 (○) and co-perfusion of ZD7288 and CRF (●) against the membrane potential (↑ shown in C, n = 4).

Figure 7. The CRF-induced augmentation in $I_{\text{H}}$ was blocked by α-helCRF but not by the aSvg-30. A, In the presence of BaCl$_2$ (100 µM), the current traces were elicited by 1-s -120 mV hyperpolarizing voltage steps (Vh = -50 mV) under ACSF, 300 nM CRF, and 1 µM α-helCRF co-perfused with 300 nM CRF. B, Plots of Iss in ACSF (○), during the application of CRF (●), and during the co-application of α-helCRF and CRF (◊) against the membrane potential (↑ shown in A, n = 6). C, In the presence of BaCl$_2$ (100 µM), the current traces are illustrated in A under ACSF, 300 nM CRF, and 200 nM aSvg co-perfused with 300 nM CRF. D, Plots of Iss in ACSF (○), during the application of CRF (●), and during co-perfusion of aSvg-30 and 300 nM CRF (□) against the membrane potential (↑ shown in C, n = 6). E, Plots of the increased-$I_{\text{H}}$ in the application of 300 nM CRF (○) and the co-perfusion of 1 µM α-helCRF and CRF (●) (n = 7). F, Plots of the increased-$I_{\text{H}}$ in the application of 300 nM CRF (○) and 200 nM aSvg-30 co-perfused with CRF (●) (n = 6). It should be noted that the CRF-induced increase in $I_{\text{H}}$ was abolished by α-helCRF but not attenuated by the CRFR-2 selective antagonist, aSvg-30.

Figure 8. Identification of CRFR-1, CRFR-2, HCN1, HCN2, HCN3, and HCN4 channel mRNA in CRF-sensitive neurons by single-cell RT-mPCR analysis. The positive control (RT+) showed that the mRNAs of CRFR-1, CRFR-2, HCN1, HCN2, HCN3, HCN4, and GAPDH were detected from the
rat hypothalamic tissue total RNA. GAPDH transcripts were analyzed in the same cells as an internal control for the RT reaction. The expected size of the PCR products is indicated, and the single-cell PCR products were verified with sequencing. In addition, a single cell and the rat hypothalamic tissue total RNA were processed without RT (-RT), but no PCR product was obtained (number 7). All six of the CRF-sensitive neurons (Cell Nos. 03101403, 03101604, 03111802, 03112104, 03112701, and 0421202) expressed CRFR-1, CRFR-2, HCN1, HCN2, and HCN3 channel mRNA but not HCN4 channel mRNA.
Figure 1
Figure 2
Figure 3
Figure 5
Figure 6
Figure 8

- HCN1: 254 bp
- HCN2: 419 bp
- HCN3: 269 bp
- HCN4: 405 bp
- CRF-R1: 394 bp
- CRF-R2: 303 bp
- GAPDH: 361 bp

Dispersed Single Cells vs. Tissue Control

bp | 1 | 2 | 3 | 4 | 5 | 6 | 7
---|---|---|---|---|---|---|---
+RT | -