Neuropeptide FF and neuropeptide VF inhibit GABAergic neurotransmission in parvocellular neurons of the rat hypothalamic paraventricular nucleus

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Am J Physiol Regul Integr Comp Physiol 292: R1872–R1880, 2007. First published February 8, 2007; doi:10.1152/ajpregu.00407.2006.——Neuropeptide FF (NPFF) and neuropeptide VF (NPVF) belong to the RFamide family of peptides that have been implicated in a wide variety of physiological functions in the brain, including central autonomic and neuroendocrine regulation. The effects of these peptides are mediated via NPFF1 and NPFF2 receptors that are abundantly expressed in the rat brain, including the hypothalamic paraventricular nucleus (PVN), an autonomic nucleus critical for the secretion of neurohormones and the regulation of sympathetic outflow. In this study, we examined, using whole cell patch-clamp recordings in the brain slice, the effects of NPFF and NPVF on inhibitory inputs to PVN neurons. Under voltage-clamp conditions, NPFF and NPVF reversibly and in a concentration-dependent manner reduced the evoked bicuculline-sensitive inhibitory postsynaptic currents (IPSCs) in parvocellular PVN neurons by 25 and 31%, respectively. RF9, a potent and selective NPFF receptor antagonist, blocked NPFF-induced reduction of IPSCs. Recordings of miniature IPSCs in these neurons following NPFF and NPVF applications showed a reduction in frequency but not amplitude, indicating a presynaptic locus of action for these peptides. Under current-clamp conditions, NPVF and NPFF caused depolarization (6–9 mV) of neurons that persisted in the presence of TTX but was abolished in the presence of bicuculline. Collectively, these data provide evidence for a disinhibitory role of NPFF and NPVF in the hypothalamic PVN via an attenuation of GABAergic inhibitory input to parvocellular neurons of this nucleus and explain the central autonomic effects of NPFF.

morphine modulatory peptide; RFamide; electrophysiology

Neuropeptide FF (NPFF) and neuropeptide VF (NPVF) belong to the family of RFamide peptides that play an important role in the control of pain and analgesia (see Refs. 30, 33). For NPFF and related peptides, two G protein-coupled receptors, designated NPFF1 and NPFF2, have been identified in human and rat central nervous system (CNS) tissues (6, 39). Binding data suggest that the NPVF is a more potent ligand for the NPFF1 receptor, whereas NPFF has a higher affinity for the NPFF2 receptor (25). The distribution of NPFF and NPVF and their receptors in the brain and spinal cord is in keeping with a wider role for these peptides than pain and analgesia (1, 11, 15, 25). Indeed, NPFF has been implicated in cardiovascular regulation, anxiety, water balance, and food intake (for review, see Ref. 29). In the brain, NPFF has been shown to influence the secretion of vasopressin from the hypothalamus and activate neurons of cardiovascular centers in the brain stem that regulate sympathetic autonomic outflow (2, 16).

Previous studies from our laboratory revealed that intracerebroventricular injections of NPFF caused a rapid dose-dependent hypertensive action (17). Several lines of evidence suggested that the hypothalamic paraventricular nucleus might be an important locus for these central cardiovascular effects of NPFF. First, using detection of the Fos protein, we observed that intracerebroventricular NPFF causes an activation of parvocellular, but not magnocellular, neurons of the hypothalamic paraventricular nucleus (PVN) (17). Little or no Fos was detected in other cardiovascular CNS centers. Second, NPFF mRNA and NPFF-like immunoreactivity are abundantly located in the hypothalamic PVN (1, 5, 38). Third, the hypothalamic PVN is enriched in NPFF binding sites and receptors (6, 11).

The PVN is a bilateral periventricular structure in the hypothalamus that contains separate populations of neurons that 1) regulate the secretion of hormones from the posterior pituitary (magnocellular neurons), 2) project to the median eminence to control adenohypophyseal hormone release (parvocellular neurons), and 3) send projections to the caudal brain stem and spinal cord autonomic centers for control of sympathetic outflow (parvocellular neurons) (36). Studies utilizing patch-clamp recordings in the hypothalamic brain slices permit a reliable identification of parvocellular neurons of the PVN from their magnocellular counterparts on the basis of their distinct electrophysiological properties (4, 26, 37). Both the parvo- and magnocellular PVN neurons receive a variety of inhibitory and excitatory synaptic inputs. The importance of inhibitory inputs to PVN neurons is highlighted by anatomical data, which show that ~50% of all synaptic connections made in the PVN are GABAergic in nature (7) and serve to mediate peripheral cardiovascular- and energy balance-related signals to this hypothalamic nucleus (27, 31). In this study, we used patch-clamp recordings in an in vitro hypothalamic slice preparation to examine effects of NPFF and NPVF on GABAergic inhibitory synaptic inputs to parvocellular PVN neurons with a view to identifying the cellular basis for the observed central cardiovascular effects of these peptides.
MATERIALS AND METHODS

Slice preparation. Male Sprague-Dawley rats (21–25 days old) were killed according to a protocol approved by the University of Alberta Health Sciences Laboratory Animal Welfare Committee. The brain was quickly removed and placed into ice-cold, carbogenated (95% O2-5% CO2) artificial cerebrospinal fluid (aCSF; pH 7.3–7.4). Coronal slices (350 μm thick) were cut from a block of forebrain tissue containing the hypothalamic PVN (21, 22) in cold (4°C), carbogenated (95% O2-5% CO2) aCSF using a vibratome. Slices were hemisected and incubated in aCSF at room temperature (22°C) for at least 1 h before recording. A slice was then transferred into a 500-μl recording chamber, where it was submerged and continuously perfused with room temperature aCSF (22°C) at a rate of 2–3 ml/min. The composition of the aCSF was (in mM) 126 NaCl, 2.5 KCl, 1.2 NaH2PO4, 1.2 MgCl2, 2.4 CaCl2, 25 NaHCO3, and 11 glucose.

Electrophysiological recording. Whole cell patch-clamp recordings (in continuous single-electrode voltage-clamp or bridge current-clamp modes) were obtained using glass micropipettes (Garner Glass; tip resistance 4–10 MΩ) with a solution containing (in mM) 140 K-glucuronate, 2 KCl, 5 HEPES, 10 EGTA, 5 Mg-ATP, and 0.5 Na-GTP, adjusted to pH 7.2–7.3 with 1 M KOH (290–320 mosM). High-resistance seals (1–3 GΩ) were obtained in PVN neurons viewed with an upright video microscope equipped with differential interference contrast-infrared optics. Signals were processed with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), filtered, and digitized using DigiData 1322 (Molecular Devices). Parvocellular PVN neurons were identified by their position in the slice and by their electrophysiological properties. Parvocellular cells were distinguished by the absence of a transient outward rectification (Ih) and the presence of a low threshold spike when depolarized from hyperpolarizing membrane potentials (14). Neurons were only studied if their holding current and access resistance remained stable for 10–15 min before any other manipulation.

Data acquisition and analysis. To study evoked inhibitory postsynaptic currents in PVN neurons, we evoked synaptic currents by electrical stimulation (200 μs; 10–40 V) through a bipolar tungsten electrode connected to a stimulator (Iso-Flex; AMPI, Jerusalem, Israel) and driven by a timer (Master-8; AMPI). Stimuli were applied using a bipolar stimulating electrode that was positioned in the ventral region of the slice immediately adjacent to the ventricle between PVN and the suprachiasmatic nucleus, a site where electrical stimulation has previously been shown to activate GABAergic inputs to PVN neurons (3). Tip of the stimulating electrode was on the average 200–800 μm from the recorded PVN neuron. During synaptic stimulation, the cells were held at −40 mV. A stimulus intensity that yielded a response 50–60% of the maximum synaptic response was used for the remainder of the experiment. To eliminate possible glutamate-mediated contamination of inhibitory synaptic responses, either 100 μM kynurenic acid (KYN) or a combination of 5 M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX: a non-NMDA receptor antagonist) and 10 μM 6,7-diamino-5-phosphonovaleric acid (APV; a NMDA receptor antagonist) was present in the aCSF at all times. At least three control synaptic responses were taken at 3-min intervals to ensure the stability of the synaptic responses before drug applications. Before, during, and after drug application, three successive synaptic responses were taken 10 s apart, digitally averaged, and stored for off-line analysis. In all experiments examining synaptic currents, a −20-mV, 100-ms duration square pulse was applied 150 ms after synaptic stimulation to monitor input and series/access resistance.

Miniature inhibitory postsynaptic currents (mIPSCs) were acquired using pCLAMP software in the presence of 1 μM tetrodotoxin (TTX) and glutamate receptor blockers (KYN or CNQX + APV) at a sampling rate of 2 kHz. The cells were held at −40 mV. A stretch of ~10 min of mIPSC activity was collected, and then NPFF or NPVF was bath-applied for 10 min (approximate time for its maximal evoked synaptic effects to occur) and mIPSCs were further collected for 10 min in the washout period. The amplitude and the inter-event interval were measured and plotted to generate amplitude and frequency distribution curves. The mean frequencies of mIPSCs in control and in the presence of NPFF or NPVF in all cells were also calculated for statistical comparisons.

All acquired data were analyzed off-line using Clampfit (Axon Instruments, Foster City, CA) or, in the case of mIPSCs, a peak detection program (Minianalysis; Synaptosoft, Leonia, NJ). Data are expressed as means ± SE in either absolute values or, as in the case of evoked IPSCs, as a percent inhibition of control responses obtained immediately before drug applications. Statistical comparisons were performed using paired or unpaired Student’s t-test or analysis of variance where appropriate. Significance was accepted at the 0.05 level.

Drug applications. All drugs were bath-applied by perfusion with aCSF containing the final concentration of the drug. NPFF and NPVF were obtained from Bachem (Torrance, CA) and Alberta Peptide Institute (Edmonton, AB, Canada). APV, bicuculline, CNQX, KYN, TTX, and aCSF components were obtained from Sigma. RF9 was supplied by Dr. F. Simonin (CNRS/INSERM, Universite de Strasbourg, France). Stock solutions of APV, bicuculline, CNQX, KYN, NPFF, NPVF, and RF9 were aliquoted and frozen at −70°C and diluted into warm, carbogenated aCSF immediately before the experiment.

RESULTS

Focal electrical stimulation in the PVN elicited mostly IPSCs in parvocellular PVN neurons. The amplitude of the evoked IPSCs was stimulus intensity dependent and reversed at between −60 and −65 mV, close to the equilibrium potential of the chloride ion (Fig. 1, A and B). Slices were treated with a combination of APV (10 μM) and CNQX (5 μM) to abolish the NMDA- and non-NMDA-mediated evoked IPSCs, and the residual NPFF-insensitive synaptic response could be completely abolished by application of bicuculline (10 μM) in the presence of glutamate receptor blockers (Fig. 1C).

Effects of NPFF and NPVF on evoked IPSC in parvocellular PVN neurons. In voltage-clamp mode when cells were held at −60 mV (close to their resting membrane potential), NPFF (5 μM) reversibly reduced the evoked IPSC without changing the holding current in 56% of cells tested (10 of 18 cells, Fig. 2A). Figure 2B1 shows the time course of the NPFF effect on the evoked synaptic response. The inhibition reversed within 15–20 min of discontinuing perfusion of the drug. NPVF (5 μM) showed a reversible inhibition of the evoked IPSC similar to that observed for NPFF in 64% of cells tested (14 of 22 cells, Fig. 2, A2 and B2). The action of NPFF and NPVF appeared to be presynaptic, since no change was observed in either the holding current (Fig. 1, A1 and A2) or the membrane current response to a voltage step (data not shown). At a dose of 5 μM, NPFF caused an average reduction of 25.0 ± 5.3% (n = 10 responsive cells, Fig. 2C) of the evoked IPSC amplitude. NPVF (5 μM) caused a reduction of 31.4 ± 4.0% (n = 14 responsive cells, Fig. 2C) in the evoked IPSC amplitude. To determine the apparent affinity of the NPFF at the presynaptic receptor, we tested NPFF in the concentration range between 0.005 and 50 μM. The NPFF-induced inhibition of the evoked IPSC was dose dependent with an EC50 of 62.5 nM (Fig. 2D). Recently, we have identified a novel potent and selective NPFF receptor antagonist, RF9, which shows a good affinity for both NPFF receptor subtypes and blocks the increase in arterial blood pressure and heart rate evoked by NPFF in rats (35).
Reversal potential of eIPSCs

neuron were recorded in the presence of 100 μM bicuculline, confirming that the synaptic events were mediated via the GABAA receptor (Fig. 3). No effect of NPFF on the decay phase of the mIPSC was observed (control: 3.37 ± 0.22 ms, NPFF: 3.46 ± 0.29 ms, recovery: 3.57 ± 0.26 ms, n = 5). These results are inconsistent with a presynaptic locus of action for NPFF.

Direct effects of NPFF and NPVF on parvocellular PVN neurons. In 24 of 47 (51%) parvocellular PVN neurons, bath application of NPFF (5 μM) or NPVF (5 μM) evoked a rapidly reversible depolarization as illustrated in Fig. 4A. In six PVN neurons, NPFF caused a depolarization of 9.3 ± 2.1 mV (mean resting membrane potential (RMP): 59.3 ± 1.1 mV). NPVF evoked a depolarization of 6.4 ± 2.5 mV (mean RMP: 62.3 ± 2.8 mV, n = 5). This depolarization usually occurred within 1 min of NPFF or NPVF reaching the slice, and the response lasted 5–10 min postdiscontinuation of the drug before returning to baseline. Cells did not show desensitization as repeat applications of NPFF or NPVF elicited similar responses. To establish whether the observed actions of NPFF and NPVF were due to direct actions of the peptides on the recorded cell or were the result of modified synaptic input from local interneurons, we recorded the effects of NPFF and NPVF in the presence of TTX (1 μM). All cells tested during TTX application continued to show depolarization in response to either NPFF ($n = 5$) or NPVF ($n = 4$) application (Fig. 4B). No significant differences were observed in the level of depolarizing response consequent to peptide applications without and with TTX (Fig. 4, C and D). To determine the role of GABAergic synaptic inputs in the excitatory effect of NPFF on PVN neurons, we tested the effect of NPFF in the presence of the GABA$A$ receptor antagonist bicuculline. In four cells that showed a response to NPFF, application of bicuculline (in the presence of TTX) resulted in a slight depolarization, which is believed to occur because of a blockade of tonic GABAergic inputs to parvocellular PVN neurons (19). However, subse-

![Figure 1](http://ajpregu.physiology.org/)
Fig. 2. NPFF and NPVF effects on eIPSCs in parvocellular PVN neurons. A1 and A2: application of NPFF (5 μM) or neuropeptide VF (NPVF; 5 μM) reversibly reduced the amplitude of focal stimulation-evoked IPSCs in a parvocellular PVN neuron. Synaptic responses are a digital average of 3 consecutive responses elicited at 10-s intervals under control conditions. NPFF or NPVF (5 μM) and washout are shown superimposed.

B1 and B2: time course of NPFF (5 μM) and NPVF (5 μM) responses. eIPSCs are normalized and averaged (NPFF, n = 10; NPVF, n = 14).

C: summary data showing reduction in peak synaptic currents following application of NPFF (n = 10 cells; 5 μM) or NPVF (n = 14 cells; 5 μM). Control eIPSCs were normalized to 100%. Data are presented as means ± SE. *P < 0.05 compared with control, paired t-test.

D: concentration-response curve for NPFF-mediated inhibition of the eIPSC in parvocellular PVN neurons. The EC50 calculated from all cells was 62.5 nM. Data are all from cells in which the NPFF effect reversed on washout (n = 2–10 cells at each concentration of NPFF).

E: RF9 (100 nM), a NPFF receptor antagonist, blocked the NPFF-induced reduction of eIPSC.
Fig. 3. Effects of NPFF on miniature IPSCs (mIPSCs) in parvocellular PVN neurons. A: representative tracings of a parvocellular PVN neuron showing mIPSCs recorded in the presence of kynurenic acid (100 μM) and tetrodotoxin (TTX; 1 μM) during control conditions, application of NPFF (5 μM), and washout. Bicuculline (10 μM) abolished mIPSCs. B and C: cumulative probability analysis of mIPSCs of the same neuron showing the distribution of the inter-event interval and peak amplitude during control, NPFF (5 μM), and washout conditions. NPFF caused a reduction in the inter-event interval of IPSCs without changing the distribution of the amplitude. D and E: summary data showing the effects of NPFF on the frequency and amplitude of mIPSCs of parvocellular PVN cells (n = 5). Data are presented as means ± SE. *P < 0.05 compared with control, paired t-test.
sequent application of NPFF (in TTX and bicuculline) failed to further increase the firing of PVN neurons as had been observed under control conditions (Fig. 4E).

**DISCUSSION**

This is the first electrophysiological study to examine the cellular effects of the RFamide group of peptides on hypothalamic parvocellular PVN neurons. We found that NPFF and NPVF, which belong to the RFamide peptide family, significantly inhibited the evoked GABAergic IPSCs in a concentration-dependent manner. NPFF-induced reduction in inhibitory synaptic responses could be abolished by RF9, a NPFF receptor antagonist, which has been recently reported to block opioid-induced hyperalgesia and elevations in blood pressure.

**Fig. 4.** Effects of NPFF on membrane potential (MP) of parvocellular PVN neurons. **A:** NPFF (5 μM) depolarized a parvocellular PVN neuron [resting MP (RMP) = −60 mV]. **B:** depolarization evoked by NPFF was preserved in the presence of TTX (1 μM; RMP = −62 mV). **C** and **D:** summary data from parvocellular PVN neurons showing the depolarization (change in MP) evoked by NPFF (5 μM) alone, NPFF with TTX (n = 6), NPVF (5 μM) alone, and NPVF with TTX (n = 5). Data are presented as means ± SE. *P < 0.05 compared with NPFF alone; **P < 0.05 compared with NPVF alone, paired t-test. **E:** parvocellular PVN depolarized by NPFF no longer responds to the peptide in the presence of TTX and bicuculline (BIC). Note the slight depolarization evoked by bicuculline suggestive of a tonic GABAergic input to the cell. **Inset** shows a histogram depicting depolarization evoked by application of NPFF (5 μM) alone and, in the same cells, NPFF in the presence of TTX and bicuculline (n = 4). *P < 0.01.
and heart rate evoked by NPFF (35). Also, NPFF and NPVF decreased the frequency of GABAergic mIPSCs without affecting the amplitude or decay time constant of mIPSCs, indicating a presynaptic locus for the actions of these peptides. Furthermore, we observed that NPFF and NPVF caused depolarization of parvocellular PVN neurons. This excitatory effect was present in TTX but eliminated in the presence of bicuculline (and TTX). Collectively, these results indicate that NPFF and the related peptide NPVF increase the excitability of parvocellular PVN neurons by disinhibiting the GABAergic synaptic input to these cells, an effect that is likely mediated by an activation of NPFF presynaptic receptors. The depolarization that we observed in the presence of bicuculline suggests the presence of tonic GABAergic inhibition of parvocellular PVN neurons. Low ambient concentrations of GABA can persistently activate certain subtypes of GABA receptors that are remote from the synapses, and such extrasynaptic receptors are implicated in mediating tonic inhibition of neurons in certain CNS regions (9). In the hippocampus, pharmacological blockade of tonic GABAergic inhibition enhances the excitability of interneurons (34). Thus NPFF inhibition of the GABAergic input may, in a similar manner, contribute to the increased excitability of parvocellular PVN neurons and involve extrasynaptic GABA receptors.

Parvocellular neurons of the hypothalamic PVN comprise a large proportion of the nucleus and include cells that project to the median eminence for control of adenohypophysal hormone secretion and preautonomic neurons projecting to the brain stem and spinal cardiovascular centers that regulate sympathetic outflow (36). A network of GABAergic interneurons located within the anterior hypothalamic area immediately ventral to the PVN provides a dense synaptic input to the PVN (32), which mediates a number of important inhibitory cardiovascular inputs to this nucleus (19). NPFF injected intracerebroventricular preferentially activates parvocellular neurons of the PVN that project to the brain stem, an effect that is accompanied by transient increases in arterial blood pressure, reflecting increased sympathetic drive (17). On the basis of this observation, we therefore hypothesized that, at a cellular level, NPFF would increase the activity of parvocellular PVN neurons, possibly via attenuation of the dense GABAergic synaptic input to these cells. Indeed, this is precisely what we observed as NPFF evoked a disinhibition of GABAergic synaptic input and an overall increase in the excitability and depolarization of parvocellular PVN neurons. The locus of NPFF effects is presynaptic on the GABAergic terminals as suggested by its effects on the frequency, but not amplitude, of the mIPSCs. Furthermore, the depolarizing effects of NPFF were abolished in the presence of bicuculline, thus providing additional evidence that the suppression of GABAergic inhibitory input is ultimately involved in the excitatory effects of NPFF on parvocellular PVN neurons. Although we did not perform specific chemical identification of the neurons we recorded from, our previous data indicate that a vast majority of intracerebroventricular NPFF-activated cells in the parvo- cellular PVN are oxytocin neurons that project to the brain stem nucleus of the solitary tract (17). Interestingly, we have recently reported that in magnocellular neurons of the PVN, NPFF increases the frequency, but not the amplitude, of bicuculline-sensitive IPSCs (18). This increased inhibitory synaptic drive to these cells is presumed to occur via a GABA-synthe-
PVN. NPFF and NPVF significantly attenuated GABAergic synaptic input to parvocellular PVN neurons through activation of presynaptic NPFF receptors. In addition, NPFF increased the excitability of parvocellular PVN neurons, and such an effect was eliminated in the presence of bicuculline. This observation further supports the notion that modulation of the GABAergic interneuron is critical in control of NPFF-evoked excitation of parvocellular PVN. The cellular actions of NPFF in the PVN that we describe presently are best understood in the context of the central cardiovascular effects of this peptide on arterial blood pressure. However, a number of other important autonomic effects of NPFF involving the hypothalamic PVN, such as those on feeding behavior, body fluid balance, and stress responses, may also be explained on the basis of the synaptic actions of NPFF and NPVF that we have reported.

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