VR₁ Receptor Activation Induces Glutamate Release and Postsynaptic Firing in the Paraventricular Nucleus

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Li, De-Pei, Shao-Rui Chen, and Hui-Lin Pan. VR₁ receptor activation induces glutamate release and postsynaptic firing in the paraventricular nucleus. J Neurophysiol 92: 1807–1816, 2004. First published April 28, 2004; 10.1152/jn.00171.2004. Neurons in the paraventricular nucleus (PVN) are important in regulating autonomic function through projections to the brain stem and spinal cord. Although the vanilloid receptors (VR₁) are present in the PVN, their physiological function is scarcely known. In this study, we determined the role of VR₁ receptors in the regulation of synaptic inputs and the excitability of spinally projecting PVN neurons. Whole cell patch-clamp recordings were performed on the PVN neurons labeled by a retrograde fluorescence tracer injected into the thoracic spinal cord of rats. Capsaicin significantly increased the frequency of glutamatergic miniature excitatory postsynaptic currents (mEPSCs) without changing the amplitude and decay time constant of mEPSCs. On the other hand, capsaicin had no effect on GABAergic miniature inhibitory postsynaptic currents (mIPSCs). The effect of capsaicin on mEPSCs was abolished by a specific VR₁ antagonist, iodo-resiniferatoxin (iodo-RTX), or ruthenium red. Importantly, iodo-RTX per se significantly reduced the amplitude of evoked EPSCs and the frequency of mEPSCs. Removal of extracellular Ca²⁺, but not Cd²⁺ treatment, also eliminated the effect of capsaicin on mEPSCs. Furthermore, capsaicin caused a large increase in the firing rate of PVN neurons, and such an effect was abolished in the presence of ionotropic glutamate receptor antagonists. Additionally, the double-immunofluorescence labeling revealed that all of the VR₁ immunoreactivity was colocalized with a presynaptic marker, synaptophysin, in the PVN. Thus this study provides the first evidence that activation of VR₁ receptors excites preautonomic PVN neurons through selective potentiation of glutamatergic synaptic inputs. Presynaptic VR₁ receptors and endogenous capsaicin-like substances in the PVN may represent a previously unidentified mechanism in hypothalamic regulation of the autonomic nervous system.

INTRODUCTION

The hypothalamic paraventricular nucleus (PVN) is a critical site for neuroendocrine control and homeostasis (Swanson and Sawchenko 1983). The PVN is a heterogenous structure containing interneurons and output neurons projecting to brain stem autonomic centers and the sympathetic preganglionic neurons located in the intermediolateral (IML) cell column of the spinal cord (Ranson et al. 1998; Shafton et al. 1998; Toth et al. 1999). There is strong anatomical and electrophysiological evidence showing that the PVN is important in the regulation of sympathetic outflow, especially during stress and certain types of hypertension (Allen 2002; de Wardener 2001; Miyakubo et al. 2002; Ranson et al. 1998). Both GABA and glutamate are two prominent inhibitory and excitatory neurotransmitters in the PVN, and the firing activities of these PVN output neurons are finely tuned by these synaptic inputs (Boudaba et al. 1997; Hermes et al. 1996; Li et al. 2002, 2003). However, the mechanisms governing glutamatergic and GABAergic inputs in the PVN are not fully known.

The capsaisin vanilloid receptor-1 (VR₁, also known as TRPV1 channel) in the primary sensory neurons is best known as a molecular sensor for nociception (Caterina et al. 1997). The VR₁ receptors are characterized as a nonselective cation channel that belongs to the transient receptor potential family (Gunthorpe et al. 2002). Activation of VR₁ receptors produces an inward current carried by nonselective cations with high permeability for divalent cations such as Ca²⁺ (Caterina et al. 1997; Liu and Simon 1994). Recent studies have shown that the VR₁ mRNA is present in several regions of the CNS (Mezey et al. 2000). Also, the VR₁ immunoreactivity and the binding sites of the VR₁-selective radioligand, [³H]resiniferatoxin, are located in the brain regions including the hypothalamus and locus coeruleus (Mezey et al. 2000; Szabo et al. 2002).

The potential physiological role of VR₁ receptors in the PVN remain unclear. Recent studies suggest that capsaicin enhances glutamatergic synaptic transmission in the locus coeruleus and substantia nigra through activation of VR₁ receptors (Marinelli et al. 2002, 2003). However, the role of VR₁ receptors in the control of excitatory and inhibitory synaptic inputs and the excitability of PVN output neurons has not been studied. Therefore in this study, we combined in vivo retrograde labeling and in vitro whole cell recording techniques in the hypothalamic slice to test the hypothesis that VR₁ receptor activation excites spinally projecting PVN neurons through potentiation of glutamatergic synaptic inputs.

METHODS

Retrograde labeling of spinally projecting PVN neurons

Sprague-Dawley rats (6–8 wk old; Harlan, Indianapolis, IN) of either sex were used for this study. The surgical preparations and experimental protocols were approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine and conformed to the National Institutes of Health guidelines on the ethical use of animals. The spinal cord at the T1–T4 level was exposed through dorsal laminectomy under halothane anesthesia. A suspension...
of rhodamine-labeled fluorescent microspheres (Fluosphere, 0.04 μm; Molecular Probes, Eugene, OR) was pressure-ejected (Picospritzer II; General Valve, Fairfield, NJ) bilaterally into the region of the IML of the spinal cord in 2 or 3 separate 50-nl injections using a glass micropipette (20- to 30-μm tip diameter). The pipette was positioned with a micromanipulator at about 500 μm below the dorsolateral sulcus, and the tracer injection was monitored through a surgical microscope (Li et al. 2002, 2003). The muscles were sutured and the wound was closed after injection. Animals were returned to their cages for 3–7 days, which is sufficient time to permit retrograde tracer being transported to the PVN. The rats were inspected daily for motor activity, signs of infection, and food and water intake to assess the health status of the animals.

Slice preparations

Three to 7 days after the tracer injection, the rats were rapidly decapitated under halothane anesthesia. The brain was quickly removed and placed in ice-cold artificial cerebral spinal fluid (aCSF) saturated with 95% O2 and 5% CO2. A tissue block containing the hypothalamus was cut from the brain and glued onto the stage of the vibratome (Technical Products International, St. Louis, MO), as we described previously (Li et al. 2002, 2003). Coronal slices (300 μm in thickness) containing the PVN were cut from the tissue block in the ice-cold aCSF. The slices were preincubated in aCSF, which was continuously gassed with 95% O2 and 5% CO2 at 34°C for 1 h until they were transferred to the recording chamber. The perfusion solution contained (in mM): 124.0 NaCl, 3.0 KCl, 1.3 MgSO4, 2.4 CaCl2, 1.4 NaH2PO4, 10.0 glucose, and 26.0 NaHCO3. For the Ca2+-free solution, CaCl2 was removed and replaced with an equimolar concentration of CoCl2 in the perfusion solution.

Recordings of postsynaptic currents and firing activity of labeled-PVN neurons

Recordings of postsynaptic currents and neuronal activity were performed in a radio frequency–shielded room using the whole cell voltage- and current-clamp technique, as we described previously (Li et al. 2002, 2003). The recording pipettes were triple-pulled using borosilicate glass capillaries (1.2 mm OD, 0.86 mm ID; World Precision Instruments, Sarasota, FL). The resistance of the pipette was 4–6 MΩ when it was filled with the internal solution containing (in mM): 130.0 potassium gluconate, 1.0 MgCl2, 10.0 HEPES, 10.0 EGTA, 1.0 CaCl2, and 4.0 ATP-Mg; adjusted to pH 7.25 with 1 M KOH (280–300 mOsm). The slice was placed in a glass-bottomed chamber (Warner Instruments, Hamden, CT) and fixed with a grid of parallel nylon threads supported by a U-shaped stainless steel weight. The slice was perfused at 3.0 ml/min at 34°C maintained by an in-line solution heater and a temperature controller (model TC-324; Warner Instruments). It took about 1 min to completely exchange the solution inside the recording chamber at the perfusion rate of 3 ml/min. Whole cell recordings from labeled PVN neurons were performed under visual control using a combination of epifluorescence illumination and differential interference contrast (DIC) optics on an upright microscope (BX50WI, Olympus, Tokyo, Japan). The fluorescence-labeled neurons located in the medial one third of the PVN area, between the third ventricle and the fornix, were selected for recording (Li et al. 2002, 2003). The labeled neuron was briefly identified with the aid of epifluorescence illumination. A tight gigaseal was subsequently obtained in the labeled neuron viewed with the DIC optics (Fig. 1).

Recordings of postsynaptic currents began 5–10 min later after the whole cell access was established and the recording reached a steady state. Signals were processed with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). A liquid junction potential of ~15.3 mV (for the potassium gluconate pipette solution) was corrected during off-line analysis. Signals were filtered at 1–2 kHz, digitized at 10 kHz using Digidata 1322 (Axon Instruments), and saved to a hard drive of a computer. The miniature excitatory postsynaptic currents (mEPSCs) were recorded in the presence of 1 μM tetrodotoxin (TTX) and 20 μM bicuculline (a GABA A receptor antagonist) at a holding potential of ~70 mV. The miniature inhibitory postsynaptic currents (mIPSCs) were recorded in the presence of 1 μM TTX, 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, a non-NMDA receptor antagonist), and 50 μM 2-amino-5-phosphonopentanoic acid (AP5, an NMDA receptor antagonist) at a holding potential of 0 mV (Li et al. 2002, 2003).

To study the effect of capsaicin on evoked EPSCs (eEPSCs) in labeled PVN neurons, synaptic currents were evoked by electrical stimulation (0.2 ms, 0.4–0.8 mA, and 0.2 Hz) through a bipolar tungsten electrode connected to a stimulator (Grass Instruments, Quincy, MA). The tip of the stimulating electrode was placed 200–600 μm away from the recorded neuron inside the PVN proper (Li et al. 2003). To avoid recording of eEPSCs elicited at the supramaximal stimulating intensity, we reduced the initial stimulation current so that the amplitude of eEPSCs was about 70–80% of maximal eEPSCs. A sodium channel blocker, lidocaine N-ethyl bromide quaternary salt (QX-314, 10 mM) was included in the pipette solution to block the Na+ currents and spontaneous action potentials in these voltage-clamp experiments. Based on the optimal reversal potentials determined for CNQX-sensitive EPSCs, the eEPSCs was recorded at a holding potential of ~70 mV.

The spontaneous firing activity of labeled PVN neurons was recorded using the whole cell current-clamp technique (Li et al. 2002, 2003). The recording procedures were similar to those used for postsynaptic current recordings as described above except that TTX and QX-314 were not used. Recordings of the firing activity of labeled PVN neurons began about 5 min after the whole cell access was established and the firing activity reached a steady state. Signals were processed, recorded, and analyzed as described above.

Capsaicin, iodo-resiniferatoxin (iodo-RTX), ruthenium red, CNQX, and bicuculline were obtained from Sigma (St. Louis, MO). TTX and QX-314 were purchased from Alomone Labs (Jerusalem, Israel). The concentrations of capsaicin, ruthenium red, CNQX, and iodo-RTX were derived from previous studies (Li et al. 2003; Marinelli et al. 2002, 2003; Trudeau et al. 1996) and tested in the pilot experiments. All the drugs were prepared immediately before the experiments and applied to the recording chamber using syringe pumps.
Double-immunofluorescence labeling of VR1 receptors and synaptophysin in the PVN

To determine whether the VR1 receptors are located presynaptically in the PVN, sections from the hypothalami were immunostained for colocalization of the VR1 receptor and synaptophysin (Li et al. 2003; Pan et al. 2003), a specific marker for presynaptic terminals, in 3 separate rats. Under deep anesthesia with sodium pentobarbital (60 mg/kg, ip), rats were intracardially perfused with 200 ml of ice-cold normal saline containing 1,000 units of heparin followed by 500 ml of 4% paraformaldehyde in 0.1 M PBS (pH 7.4) and then 200 ml of 10% sucrose in 0.1 M PBS (pH 7.4). The brain was removed quickly and postfixed for 2 h in the same fixative solution and cryoprotected in 30% sucrose in PBS for 48 h at 4°C. The sections were cut to a thickness of 25 μm and collected free floating in 0.1 M PBS. For VR1 and synaptophysin double-immunofluorescence labeling, the labeling intensity of the first primary antibody (rabbit anti-VR1, N terminal polyclonal IgG antibody, dilution 1:1000; Neuromics, Minneapolis, MN) was enhanced with TSA (tyramide signal amplification, and conventional immunofluorescent labeling was then performed with the second primary antibody (mouse anti-synaptophysin). To perform the TSA labeling, sections were first incubated in 3% H2O2 with 10% of methanol to quench endogenous peroxidase and blocked in TNB (0.1 M Tris-HCl, 0.15 M NaCl, and 0.5% blocking reagent) for 2 h at room temperature. After rinsing, the sections were mounted on glass slides, dried, and coverslipped. The sections were then viewed using a confocal microscope (Leica, Wetzlar, Germany), and the areas of interest were photographed. Confocal laser scanning microscopy was used for accurate colocalization of fluorescent markers, because the thin (~0.3 μm) optical sectioning generated by the confocal microscope eliminates the confounding effect of out-of-focus fluorescence. In the higher magnification images, the colocalization was indicated by the color change (yellow) and represents colocalization (Li et al. 2002, 2003).

Data analysis

Data are presented as means ± SE. To determine the amplitude of the eEPSCs, 20 consecutive eEPSCs were averaged and measured using pClamp 9.0 analysis software. The mEPSCs, mIPSCs, and the firing activity were analyzed off-line with a peak detection program (MiniAnalysis, SynaptoSoft, Leonia, NJ). Detection of events was accomplished by setting a threshold above the noise level. The distribution cumulative probability of the amplitude and interevent interval of mEPSCs and mIPSCs was compared using the Komgorov–Smirnov test, which estimates the probability that 2 cumulative distributions are similar. The decay time constant of mEPSCs or mIPSCs was obtained by fitting the decay phase of synaptic currents with exponential equation; ≥10 mEPSCs and mIPSCs were used in each analysis. For cells displaying intermittent firing activity, the membrane potential was measured when the cell was silent and the membrane potential became stable. For those cells showing tonic activity, the membrane potential was usually estimated 200 ms before initiation of the action potential. The effects of drugs on the firing activity, peak amplitude of eEPSCs, and amplitude and frequency of mIPSCs and mEPSCs were determined by Wilcoxon signed-rank test or nonparametric ANOVA (Kruskal–Wallis) with Dunn’s post hoc test. P < 0.05 was considered to be statistically significant.

Results

Whole cell patch-clamp recordings were performed on 97 PVN cells (n = 39 rats) labeled by Fluospheres. Because of the desensitizing effect of capsaicin, only one cell was studied in each brain slice. The spinal cords were taken out after sacrificing the rats and viewed under a microscope equipped with epifluorescence to ensure that the injection and diffusion sites of the tracer were located in and around the IML in the spinal cord (Li et al. 2003, 2004). The labeled PVN neurons displayed a resting membrane potential ranging from −72.8 to −55.9 (−60.7 ± 6.8) mV, an input resistance between 360 and 625 (432.7 ± 32.7) MΩ, and an amplitude of action potentials >60 mV.

Excitatory effect of capsaicin on glutamatergic EPSCs of labeled PVN neurons

To determine the effect of capsaicin on glutamatergic synaptic inputs to labeled PVN neurons, we examined the effect of capsaicin on the spontaneous mEPSCs. The mEPSCs were recorded in the presence of 1 μM TTX and 20 μM bicuculline. Capsaicin, in a concentration of 1 μM, significantly increased the frequency of mEPSCs from 3.85 ± 0.77 to 11.70 ± 1.30 Hz (398 ± 70% increase from the control, P < 0.05) without altering the amplitude and the decay time constant of mEPSCs in 12 labeled neurons tested (Fig. 2). The capsaicin took effect within 4.98 ± 0.37 min after application, and the effect of capsaicin subsided within 2–5 min as a result of rapid desensitization of VR1 receptors. In each of these 12 cells, the cumulative probability analysis revealed that the distribution pattern of the interevent interval of mEPSCs shifted to the left in response to capsaicin administration, whereas the distribution pattern of the amplitude of mEPSCs was not changed (Fig. 2, B and C). The decay time constant of mEPSCs was best fitted by a single-exponential function (Fig. 2D). The decay phase of mEPSCs during control was not significantly different from that during application of capsaicin (2.67 ± 0.63 vs. 2.83 ± 0.52 ms; P > 0.05; n = 12). The mEPSCs were completely abolished by perfusion of 20 μM CNQX in all 8 cells tested (Fig. 2A).

Furthermore, the effect of capsaicin on evoked EPSCs was tested in the presence of 20 μM bicuculline. In 5 separately labeled PVN neurons, application of 1 and 10 μM capsaicin did not significantly alter the amplitude of evoked EPSCs (238.5 ± 26.9 vs. 219.4 ± 28.5 pA at 1 μM and 208.7 ± 31.5 pA at 10 μM).

Role of endogenous VR1 activation in regulation of glutamatergic EPSCs

To determine whether the glutamatergic inputs to PVN neurons was tonically influenced by endogenous VR1 activation, the effect of a specific VR1 receptor antagonist, iodo-RTX (Wahl et al. 2001), on mEPSCs and evoked EPSCs was tested.
In 8 labeled PVN neurons, iodo-RTX (300 nM) alone decreased the frequency of mEPSCs from 3.38 ± 0.5 to 2.11 ± 0.3 Hz (P < 0.05, Fig. 3, A–F) without affecting the amplitude and decay time constant of mEPSCs. The inhibitory effect of iodo-RTX on the frequency of mEPSCs was no longer present after 20–30 min washout. Furthermore, 300 nM iodo-RTX significantly decreased the peak amplitude of evoked EPSCs from 226.1 ± 28.7 to 136.3 ± 22.6 pA (P < 0.05, Fig. 3, G and H) in another 7 PVN neurons.

Role of VR1 receptors in the effect of capsaicin on mEPSCs

To confirm that the effect of capsaicin on mEPSCs was mediated through VR1 receptors, iodo-RTX was applied before perfusion of capsaicin. Capsaicin failed to increase the frequency of mEPSCs in the presence of 300 nM iodo-RTX in 11 labeled PVN neurons (Fig. 4, A–E). Also, to determine whether the effect of capsaicin on mEPSCs was through activation of the VR1 nonselective cation channels, ruthenium red, a dye that exhibits properties of noncompetitive antagonism for VR1 channels (Nagy and Rang 1999), was used. Ruthenium red (10 μM) alone produced a brief increase in the frequency of mEPSCs, which lasted for 3–5 min. This effect of ruthenium red is largely attributed to the rapid and reversible neurotransmitter release induced by a transient rise in intraterminal Ca2+ (Congar and Trudeau 2002; Trudeau et al. 1996). Subsequent capsaicin application failed to increase the frequency of mEPSCs in the presence of ruthenium red in 7 labeled PVN neurons (Fig. 4, F and G).

Role of extracellular Ca2+ and Ca2+ channels in the effect of capsaicin on mEPSCs

To determine the role of voltage-dependent Ca2+ channels in the capsaicin-induced increase in mEPSCs, a general voltage-dependent Ca2+ channel blocker, Cd2+, was used in another 7 labeled PVN neurons. Pretreatment with Cd2+ (50–100 μM) had no significant effect on the frequency and amplitude of mEPSCs of labeled PVN neurons. Capsaicin significantly increased the frequency of mEPSCs from 4.37 ± 0.8 to 9.15 ± 1.9 Hz (213 ± 17% increase from the control, P < 0.05) in the presence of Cd2+ without altering the amplitude and the decay time constant of mEPSCs (Fig. 5, A–E). The effect of capsaicin on increased frequency of mEPSCs in normal aCSF (398 ± 70%) was significantly greater than that in the presence of Cd2+ (213 ± 17%, P < 0.05, Student’s unpaired t-test).

To further determine whether the capsaicin-induced increase in the frequency of mEPSCs was dependent on extracellular Ca2+, the effect of capsaicin on mEPSCs was tested in a Ca2+-free aCSF. In this experiment, CaCl2 was removed and replaced by an equimolar concentration of CoCl2 in the perfusion solution. The frequency and the amplitude of mEPSCs was not significantly affected by perfusion of the Ca2+-free aCSF. Subsequent application of capsaicin (1 μM) did not alter the frequency (from 4.18 ± 1.2 to 4.28 ± 1.1 Hz, P > 0.05) and the amplitude of mEPSCs in the Ca2+-free aCSF in 8 labeled PVN neurons (Fig. 5, F and G).

Effect of capsaicin on the GABAergic mIPSCs

The spontaneous mIPSCs were recorded from 7 labeled PVN neurons in the presence of 1 μM TTX and 20 μM CNQX. Neither the frequency nor the amplitude of mIPSCs was affected by bath application of 1 and 10 μM capsaicin (Fig. 6). The effect of 10 μM capsaicin on mIPSCs was further analyzed by measuring the time constant of the decay phase of mIPSCs. The decay phase of mIPSCs was best fitted by a double-exponential function equation (Fig. 6D). Neither the fast (7.68 ± 0.58 vs. 6.39 ± 0.48 ms) nor the slow (27.21 ±
was best
superimposed averages of 100 consecutive mEPSCs obtained during control and iodo-RTX application. Decay phase of mEPSCs
interevent interval (B) during control, iodo-RTX application, and washout. Iodo-RTX increased the
C) and peak amplitude (D) of mEPSCs of 8 labeled PVN neurons. G: original recordings showing that iodo-RTX decreased the peak amplitude of eEPSCs. Data are presented as means ± SE. *P < 0.05, compared with the control (Kruskal–Wallis ANOVA followed by Dunn’s post hoc test). I-RTX: iodo-RTX.

2.87 vs. 28.59 ± 3.02 ms) component of the decay phase of
mIPSCs during capsaicin application was significantly different from those during the control. Application of 20 μM
bicuculline completely abolished the mIPSCs in these 7 cells (Fig. 6).

Presynaptic location of VR₁ receptors in the PVN
To determine the presynaptic location of VR₁ receptors, double-immunofluorescence staining was performed using specific antibodies against synaptophysin and VR₁ receptors in the same brain section. All negative controls (omitting primary antibodies) displayed no detectable staining. Almost all of the synaptophysin immunoreactivity in the PVN occurred in the form of fine punctate deposits that often outlined neuronal cell bodies in a basketlike fashion (Fig. 7A). From the confocal images, numerous puncta immunoreactive for VR₁ receptor (green) were present extensively in the PVN (Fig. 7B). All VR₁ receptor immunoreactivities were colocdized with synaptophysin, as indicated by the color change (yellow, Fig. 7C).

Effect of capsaicin and glutamate antagonists on the firing activity of labeled-PVN neurons
Because capsaicin preferentially increased the excitatory glutamatergic synaptic inputs to labeled PVN neurons, we reasoned that the excitability of these neurons would be increased by capsaicin. To directly test this hypothesis, the effect of 1 μM capsaicin on the spontaneous firing activity of labeled PVN neurons was determined using whole cell current-clamp recordings. The majority (21/26, 81%) of labeled PVN neurons recorded displayed spontaneous activity. In 11 PVN neurons tested, 1 μM capsaicin significantly increased the firing rate from 2.76 ± 0.82 to 6.28 ± 0.14 Hz (P < 0.05, Fig. 8) after an onset latency of 5.22 ± 0.41 min. The membrane potential was slightly, but not significantly, depolarized from −70.3 ± 1.47 to −67.8 ± 1.49 mV by capsaicin.

To determine the role of glutamatergic synaptic inputs in the excitatory effect of capsaicin, the effect of 1 μM capsaicin on the firing activity of PVN neurons was tested after treatment with glutamate NMDA and non-NMDA antagonists, AP5 and CNQX. In 6 labeled-PVN neurons, the spontaneous activity was not significantly altered by perfusion of 20 μM CNQX. Subsequent perfusion of 1 μM capsaicin only slightly increased the firing rate of these cells from 2.89 ± 1.35 to 3.58 ± 1.9 Hz (P > 0.05). In another 9 labeled PVN neurons, the firing activity was not significantly changed after treatment with 20 μM CNQX plus 50 μM AP5. Subsequent perfusion of 1 μM capsaicin failed to increase the firing activity of these 9 PVN neurons in the presence of CNQX and AP5 (2.51 ± 0.98 vs. 2.63 ± 0.83 Hz, P > 0.05, Fig. 8).
DISCUSSION

This is the first study investigating the potential physiological function of VR1 receptors in the PVN. We found that capsaicin significantly increased the frequency of glutamatergic mEPSCs without affecting the amplitude and decay time constant of mEPSCs in spinally projecting PVN neurons. Our data are consistent with a neurochemical study showing that capsaicin stimulates glutamate release from hypothalamic slices (Sasamura et al. 1998). The effect of capsaicin on glutamatergic synaptic transmission has been shown recently in several brain regions including the nucleus tractus solitarius, locus coeruleus, and the substantia nigra (Doyle et al. 2002; Marinelli et al. 2002, 2003). We observed that

Although the VR1 immunoreactivity and mRNA are present in the PVN, its physiological function and precise cellular localization have not been studied. In the present study, we found that capsaicin preferentially increased the frequency of glutamatergic mEPSC without altering the amplitude and decay time constant of mEPSCs in spinally projecting PVN neurons. Our data are consistent with a neurochemical study showing that capsaicin stimulates glutamate release from hypothalamic slices (Sasamura et al. 1998). The effect of capsaicin on glutamatergic synaptic transmission has been shown recently in several brain regions including the nucleus tractus solitarius, locus coeruleus, and the substantia nigra (Doyle et al. 2002; Marinelli et al. 2002, 2003). We observed that
Capsaicin receptors and synaptic transmission

**FIG. 6.** Lack of effect of capsaicin on mIPSCs in labeled PVN neurons. A: representative tracings from a labeled neuron in the PVN showing spontaneous mIPSCs during control and application of 10 μM capsaicin and 20 μM bicuculline. B and C: cumulative plot analysis of mIPSCs of the same neuron showing the distribution of the interevent interval (B) and amplitude (C) during control and application of 10 μM capsaicin. Neither the interevent interval nor the amplitude of mIPSCs was affected by capsaicin. D: superimposed averages of 100 consecutive mIPSCs obtained during control and capsaicin application. Decay phase of mIPSCs was best fitted with a double-exponential equation. Both τ̇est (7.68 vs. 7.37 ms) and τ̇slow (28.21 vs. 29.69 ms) of the decay phase during control and capsaicin application were similar. E and F: summary data showing the effect of capsaicin on the frequency (E) and amplitude (F) of mIPSCs of 7 labeled PVN neurons. Data are presented as means ± SE. *P < 0.05, compared with the control (Kruskal–Wallis ANOVA, followed by Dunn’s post hoc test). Caps: capsaicin.

capsaicin did not potentiate but even slightly reduced the amplitude of evoked EPSCs in labeled PVN neurons. This finding is consistent with previous studies showing that capsaicin has no consistent effects on evoked EPSCs in the substantia nigra and locus coeruleus (Marinelli et al. 2002, 2003). The reasons for lack of a potentiating effect of capsaicin on evoked EPSCs are not clear. One possibility is that the presynaptic terminals being activated by electrical stimulation are a different population from those being stimulated by capsaicin. We are unable to identify and stimulate specific afferent pathways to labeled PVN neurons in this slice preparation. Another possibility is that an excessive direct depolarization of the presynaptic terminals by capsaicin can interfere with action potential–evoked glutamate release resulting from a “conduction block” (Yang et al. 1999). Our immunocytochemistry experiment provides additional complementary evidence that VR1 receptors are localized at the presynaptic terminals in the PVN. Notably the VR1 immunoreactivity was not systematically examined in the PVN in a previous study (Mezey et al. 2000). We used an enhanced immunolabeling technique in this study, which enabled us to visualize the VR1 immunoreactivity on the presynaptic terminals in the PVN. Previous studies suggest that the glutamatergic innervation of the PVN may originate from multiple sources of the CNS including the subnuclei within the hypothalamus, lateral septum, bed nucleus of the stria terminalis, and amygdala (Boudaba et al. 1997; Csaki et al. 2000; Cui et al. 2001). Our electrophysiological and immunocytochemistry data strongly suggest that capsaicin increases the synaptic glutamate release probability, and the most likely site of action is at the presynaptic glutamatergic terminals.

The effect of capsaicin on mEPSCs on spinally projecting PVN neurons was mediated by VR1 receptors, given that the specific VR1 receptor antagonist, iodo-RTX, completely abolished the capsaicin-induced increase in the frequency of mEPSCs. Because of its high binding affinity to VR1 receptors, the potent VR1 antagonist, iodo-RTX, was selected in this study (Wahl et al. 2001). The utility of capsazepine, the first known competitive VR1 antagonist, is hindered by its moderate potency (Bevan et al. 1992). At micromolar concentrations necessary to inhibit capsaicin-evoked responses, capsazepine displays nonspecific effects, including block of voltage-gated calcium channels (Docherty et al. 1997) and nicotinic receptors (Wardle et al. 1997). Furthermore, the existence of capsazepine-insensitive VR1 has been reported in rat trigeminal ganglion neurons (Liu et al. 1998). The capsaicin-induced increase in the frequency of mEPSCs was also blocked by ruthenium red, a dye that exhibits properties of noncompetitive antagonism for the nonselective cation channel. Although it has a poor selectivity, ruthenium red can effectively block the

**FIG. 7.** Confocal images showing colocalization of synaptophysin and vanilloid receptor-1 (VR1) receptor immunoreactivities in the PVN. Synaptophysin (A, red) and VR1 receptor (B, green) immunoreactivities in the PVN viewed under a confocal microscope. C: digitally merged images from A and B. Note that all VR1 receptor immunoreactivities were colocated with synaptophysin, as indicated by the color change (yellow). Scale bar, 20 μm. Images are in all cases single confocal optical sections.

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transmembrane cation current (Nagy and Rang 1999). These data suggest that the capsaicin-induced glutamate release is through activation of VR₁ receptors on presynaptic nerve terminals in the PVN.

In contrast to its action on glutamatergic mEPSCs, capsaicin had no significant effect on the GABAergic mIPSCs in labeled PVN neurons. Also, capsaicin appears to enhance only glutamatergic, but not GABAergic, synaptic transmission in the locus coeruleus and substantia nigra (Marinelli et al. 2002, 2003). The excitatory and inhibitory synaptic inputs to the PVN neurons are finely controlled by many factors. Unlike the effect of capsaicin in the PVN, both nitric oxide and angiotensin II have preferential effects on GABAergic, but not glutamatergic, synaptic inputs in the PVN (Li et al. 2002, 2003). Because activation of VR₁ receptors affects only glutamatergic, but not GABAergic, synaptic inputs to PVN neurons, it is likely that VR₁ receptors represent a previously unidentified mechanism that selectively regulates glutamatergic synaptic transmission in the PVN.

The increase in neurotransmitter release from presynaptic terminals is typically mediated through a Ca²⁺-dependent mechanism. The increase in the frequency of mEPSCs after VR₁ activation by capsaicin presumably results from an increase in intraterminal Ca²⁺ concentration through Ca²⁺ influx into the nerve terminal, in that the effect of capsaicin on mEPSCs was abolished by removal of extracellular Ca²⁺.

However, the general voltage-gated Ca²⁺ channel blocker, cadmium, failed to abolish the effect of capsaicin on mEPSCs. Thus capsaicin likely increases the glutamate release probability through calcium influx directly through the ionophore of VR₁ receptors, but not dependent on voltage-gated Ca²⁺ channels (Caterina et al. 1997; Gunthorpe et al. 2002). Nevertheless, it should be noted that the effect of capsaicin on mEPSCs in the presence of cadmium was significantly smaller than that in normal aCSF. This finding suggests that activation of VR₁ receptors increases the frequency of mEPSCs through calcium influx from both its own ionophore and the voltage-dependent Ca²⁺ channels.

Based on the observation that capsaicin preferentially increased the excitatory glutamatergic synaptic inputs to labeled PVN neurons, we hypothesized that capsaicin increases the firing activity of labeled PVN neurons through augmented glutamatergic inputs. We found that capsaicin caused a large increase in the firing activity of labeled PVN neurons. Interestingly, a long latency was observed for the effect of capsaicin on both mEPSCs and the firing activity of PVN neurons. This suggests that a second message system is likely involved in the effect of capsaicin on synaptic glutamate release. In addition to the calcium influx directly through the VR₁ receptor, a store-operated Ca²⁺ influx (Liu et al. 2003) may also play a role in the presynaptic action of capsaicin in the PVN. In the presence of blockade of NMDA and non-NMDA receptors with AP5 and CNQX, capsaicin failed to increase the firing activity of PVN neurons. Thus these data indicate that capsaicin indirectly increases the excitability of spinally projecting PVN neurons through potentiation of glutamatergic synaptic inputs. We observed that the glutamate NMDA and non-NMDA receptor antagonists per se did not significantly alter the firing activity in all the PVN neurons tested, suggesting that the basal glutamatergic inputs are probably not sufficient to alter the firing activity of PVN neurons in this slice preparation.
One important finding of the present study is that the specific VR1 antagonist, iodo-RTX, not only abolished the effect of capsaicin on mEPSCs but also reduced both the amplitude of evoked EPSCs and the frequency of mEPSCs. These data suggest that the glutamatergic synaptic input to spinally projecting PVN neurons is tonically regulated by VR1 receptors. Several capsaicin-like substances have been identified to activate or potentiate the activity of VR1 receptors. Besides protons and anandamide (Smart et al. 2000; Zygmunt et al. 1999), 12-hydroperoxyeicosatetraenoic acid and leukotriene B4 (Hwang et al. 2000; Shin et al. 2002) have been considered as endogenous VR1 agonists. Also, N-arachidonoyldopamine and N-oleylidopamine have been reported to be the endogenous agonists for VR1 receptors (Chu et al. 2003; Huang et al. 2002). At the present time, it remains unclear which of the above substances are present in sufficient concentrations that could function as the endogenous VR1 agonists in the PVN.

We recently observed that intracerebroventricular injection of capsaicin caused a profound increase in the blood pressure and renal sympathetic nerve activity in rats (unpublished data). Thus activation of VR1 receptors can increase the excitability of PVN preautonomic neurons and consequently augments the sympathetic output. These results are potentially important because the mechanism studied provides a means for selective modulation of the excitatory input to PVN preautonomic neurons. It should be emphasized that the VR1 immunoreactivity is not restricted to presynaptic terminals synapsing with spinally projecting neurons in the PVN. The VR1 receptors present in the hypothalamus most likely have other important functions including regulation of body temperature, food intake, metabolic balance, and hydromineral homeostasis. Data from the present study provide important new information about the potential physiological role and mechanisms of VR1 receptors in regulation of neuroendocrine functions.

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