Regulation of Synaptic Inputs to Paraventricular-Spinal Output Neurons by $\alpha_2$ Adrenergic Receptors

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Regulation of synaptic inputs to paraventricular-spinal output neurons by $\alpha_2$ adrenergic receptors. J Neurophysiol 93: 393–402, 2005. First published September 8, 2004; doi:10.1152/jn.00564.2004. Neurons in the paraventricular nucleus (PVN) that project to the brain stem and spinal cord are important for autonomic regulation. The excitability of preautonomic PVN neurons is controlled by the noradrenergic input from the brain stem. In this study, we determined the role of $\alpha_2$ adrenergic receptors in the regulation of excitatory and inhibitory synaptic inputs to spinally projecting PVN neurons. Excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs) were recorded using whole cell voltage-clamp techniques on PVN neurons labeled by a retrograde fluorescence tracer injected into the thoracic spinal cord of rats. Bath application of 5–20 $\mu$M clonidine, an $\alpha_2$ receptor agonist, significantly reduced the amplitude of evoked GABAergic IPSCs in a dose-dependent manner. Also, 10 $\mu$M clonidine significantly decreased the frequency (from 2.68 ± 0.41 to 1.22 ± 0.40 Hz) but not the amplitude of miniature IPSCs (mIPSCs), and this effect was blocked by the $\alpha_2$ receptor antagonist yohimbine. Furthermore, clonidine increased the paired-pulse ratio of evoked IPSCs from 1.25 ± 0.05 to 1.61 ± 0.08 ($P < 0.05$). On the other hand, clonidine had little effect on evoked glutamatergic EPSCs, mEPSCs, and the paired-pulse ratio of evoked EPSCs in most labeled cells examined. Additionally, immunofluorescence labeling revealed that the $\alpha_2A$ receptor and GABA immunoreactivities were co-localized in close apposition to labeled PVN neurons. Collectively, these data suggest that the $\alpha_2$ adrenergic receptors primarily antagonize GABAergic inputs to PVN output neurons to the spinal cord. The presynaptic $\alpha_2$ receptors function as heteroreceptors to modulate synaptic GABA release and contribute to the hypothalamic regulation of sympathetic outflow.

INTRODUCTION

The paraventricular nucleus (PVN) of the hypothalamus is an important site to integrate neuroendocrine and autonomic functions (de Wardener 2001; Swanson and Sawchenko 1983). Both anatomical and functional evidence suggests that the PVN is an important source of excitatory drive for sympathetic vasomotor tone (Allen 2002; Martin and Haywood 1992; Yang and Coote 1998). The PVN is a heterogeneous structure containing interneurons and different types of output neurons, which project to the pituitary, brain stem autonomic centers, and the preganglionic sympathetic neurons located in the intermediolateral (IML) cell column of the spinal cord (Hardy 2001; Pyner and Coote 2000; Ranson et al. 1998; Yang and Coote 1998). The PVN-spinal descending pathway plays an important role in the autonomic responses to stress and osmolarity changes in the body fluid (de Wardener 2001; Imaki et al. 1998; Swanson and Sawchenko 1983). However, the synaptic mechanisms regulating the excitability of spinally projecting PVN neurons are not fully known.

The PVN is richly innervated by noradrenergic nerve terminals originating from the brain stem, especially A1, A2, and A6 cell groups (Cunningham and Sawchenko 1988; Sawchenko and Swanson 1982). The noradrenergic inputs to the PVN and the $\alpha_2$ adrenergic receptors are involved in the control of neuronal excitability and blood pressure. For example, stimulation of noradrenergic cell groups in the medulla increases the firing activity of PVN neurons in anesthetized rats, and this effect is abolished by treatment with a noradrenergic neurotoxin, 6-hydroxydopamine (Day et al. 1984, 1985). Also, microinjection of norepinephrine into the PVN in conscious rats produces an increase in blood pressure, an effect abolished by pretreatment with idazoxan, an $\alpha_2$ receptor antagonist (Harland et al. 1989). Furthermore, microinjection of an $\alpha_2$ receptor agonist, clonidine, into the PVN increases the blood pressure in conscious rats (Ebihara et al. 1993). The cellular mechanisms regulating preautonomic PVN neurons by $\alpha_2$ adrenergic receptors remain to be determined.

The excitability of preautonomic PVN neurons is finely regulated by neurotransmitters and neuromodulators through their actions on excitatory and inhibitory synaptic inputs (Li et al. 2002, 2003, 2004b). A majority of the GABAergic and glutamatergic synaptic to the PVN neurons originate from the suprachiasmatic nucleus, subfornical organ, and other brain regions (Bains and Ferguson 1995; Cui et al. 2001; Hermes et al. 1996). Local synaptic inputs to the PVN neurons are primarily GABAergic (Boudaba et al. 1996; Tasker and Dudek 1993). However, the influence of activation of $\alpha_2$ adrenergic receptors on the GABAergic and glutamatergic synaptic inputs to the preautonomic PVN neurons has not been studied specifically. In this study, using a combination of in vivo retrograde-labeling and in vitro whole cell recordings in brain slices, we determined the role of $\alpha_2$ receptors in the control of inhibitory GABAergic and excitatory glutamatergic synaptic inputs to spinally projecting PVN neurons.

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. All efforts were made to minimize both the suffering and number of animals used. The spinal cord at the T2–T4 level was exposed through dorsal laminectomy under halothane anesthesia. A rhodamine-labeled fluorescence microsphere suspension (FluoSpheres, 0.04 μm, Molecular Probes, Eugene, OR) was pressure-ejected (Picospritzer II, General Valve Co., Fairfield, NJ) bilaterally into the region of the IML of the spinal cord in three or four separate 50-nl injections using a glass micropipette (20–30 μm tip diam). 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. 2003, 2004a). The muscles were sutured, and the wound was closed after injection. Animals were returned to their cages for 3–10 days, which is sufficient to permit retrograde tracer to be transported to the PVN. Previous studies have shown that the rhodamine microspheres do not affect the electrophysiological properties of the labeled neurons (Cui et al. 2001; Li et al. 2003, 2004a; Tseng et al. 1991). 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

Hypothalamic slices were prepared from FluoSpheres-injected rats 3–10 days after fluorescent 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) containing (in mM) 124.0 NaCl, 3.0 KCl, 1.3 MgSO4, 2.4 CaCl2, 1.4 NaH2PO4, 10.0 glucose, and 26.0 NaHCO3 saturated with 95% O2–5% CO2. A tissue block containing the hypothalamus was cut from the brain and glued onto the stage of the vibratome (Technical Product International, St. Louis, MO), as we described previously (Li et al. 2003, 2004a). The tips of the stimulating electrode were placed 200–500 μm away from the recording chamber (Li et al. 2003, 2004b). A sodium channel blocker, lidocaine N-ethyl bromide (QX-314, 10 mM), and GDP–β-s (1 mM) were included in the pipette solution to block the Na+ current and possible postsynaptic effect in these voltage-clamp experiments. Based on the optimal synaptic potentials determined for CNQX-sensitive EPSCs and bicuculline-sensitive IPSCs, the eEPSCs and eIPSCs were recorded at a holding potential of −70 mV, respectively (Li et al. 2003). To determine the effect of clonidine on paired-pulse facilitation, two synaptic responses (A1 and A2) were evoked by a pair of stimuli given at short intervals (40 ms for EPSCs and 50 ms for IPSCs). Paired-pulse facilitation was expressed as the amplitude ratio of the second synaptic response to the first synaptic response (A2/A1).

To study the evoked IPSCs and EPSCs (eIPSCs and eEPSCs) in labeled PVN neurons, synaptic currents were evoked by electrical stimulation (0.1 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 200–500 μm away from the recorded neuron within the PVN proper (Li et al. 2003, 2004b). A sodium channel blocker, lidocaine N-ethyl bromide (QX-314, 10 mM), and GDP–β-s (1 mM) were included in the pipette solution to block the Na+ current and possible postsynaptic effect in these voltage-clamp experiments. Based on the optimal synaptic potentials determined for CNQX-sensitive EPSCs and bicuculline-sensitive IPSCs, the eEPSCs and eIPSCs were recorded at a holding potential of −70 mV, respectively (Li et al. 2003). To determine the effect of clonidine on paired-pulse facilitation, two synaptic responses (A1 and A2) were evoked by a pair of stimuli given at short intervals (40 ms for EPSCs and 50 ms for IPSCs). Paired-pulse facilitation was expressed as the amplitude ratio of the second synaptic response to the first synaptic response (A2/A1).

Whole cell voltage-clamp recordings were performed to record the postsynaptic currents in a radio frequency-shielded room, as we described previously (Li et al. 2002, 2003, 2004a). The recording pipettes were triple-pulled using borosilicate capillaries (1.2 mm OD, 0.86 mm ID; World Precision Instruments, Sarasota, FL). The resistance of the pipette was ~5 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 glass-bottomed chamber. 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 (BX50 WI, Olympus, Tokyo, Japan). The fluorescence-labeled neurons located in the medial one third of the PVN were identified with the aid of epifluorescence illumination. A tight giga-Ω seal was subsequently obtained in the labeled neuron viewed with DIC optics. Recordings of postsynaptic currents began 5 min later, after the whole cell access was established and the current 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 20 kHz using Digidata 1322 (Axon Instruments), and saved to a hard drive of a computer. The miniature inhibitory postsynaptic currents (mIPSCs) were recorded in the presence of 1 μM TTX and 20 μM 6-cyano-7-nitroquinolin oxide-2,3-dione (CNQX; a glutamate non-N-methyl-D-aspartate receptor antagonist) at a holding potential of 0 mV, and the miniature excitatory postsynaptic currents (mEPSCs) were recorded in the presence of 1 μM TTX and 20 μM bicuculline at a holding potential of −70 mV. A general G protein inhibitor, guanosine 5′-O-(2-thiodiposphosphate) (GDP–β-s, 1 mM) was added into the recording pipette solution to block the possible postsynaptic action mediated by G protein activation coupled to adrenergic receptors (Li et al. 2003; Pan et al. 2002).

Recordings of postsynaptic currents of labeled PVN neurons

Immunofluorescence double labeling of α2A receptors and synaptophysin or GABA in the PVN

The α2A is the major subtype responsible for the effect of α3 receptor agonists in the brain (Bylund 1995; MacMillan et al. 1996; Tavares et al. 1996). To determine if the α2A receptors are located presynaptically in the PVN, sections from the hypothalamus were immunolabeled for co-localization of the α2A receptor and synaptophysin, a specific marker for presynaptic terminals, in three 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 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 double-labeling procedures were similar to those described previously (Li et al. 2003, 2004b). Briefly, the sections were cut in 30 μm in thickness and collected free floating in the buffer containing 0.1 M Tris-HCl and 0.15 M NaCl. For α2A, and synaptophysin double-immunofluorescence labeling, the labeling intensity of the first primary antibody (rabbit anti-α2A polyclonal IgG antibody, Neuromics, Minneapolis, MN) was enhanced with tyramide signal amplification (TSA), and conventional immunofluorescence labeling was performed with the second primary antibody (mouse anti-synaptophysin). Sections were first incubated in 1% H2O2 to quench endogenous peroxidase. Next, sections were incubated with the primary antibody (rabbit anti-α2A, dilution:1:100) for 2 h at room
temperature and 24 h at 4°C. Subsequently, sections were rinsed and incubated with biotin-SP-conjugated AffiniPure goat anti-rabbit IgG secondary antibody (dilution: 1:200, Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at room temperature. The sections were rinsed and incubated with streptavidin-horseradish peroxidase at 1:100 dilution for 30 min at room temperature. Finally, the sections were incubated with the FITC conjugated to tyramide (Perkin Elmer, Boston, MA) and incubated with the secondary primary antibody (mouse anti-synaptophysin monoclonal IgM, dilution: 1:400, Molecular Probes, according to the manufacturer’s recommendation. For the conventional GABA immunofluorescent staining of GABA, sections were rinsed and incubated with the primary antibody (mouse anti-GABA, Sigma; dilution: 1:100) for 2 h at room temperature and overnight at 4°C (Finnegan et al. 2004). Subsequently, sections were rinsed and incubated with the secondary antibody (biotin-SP-conjugated goat anti-mouse IgG, Jackson Immunoresearch; dilution: 1:200) for 2 h at room temperature. Finally, the sections were washed and incubated with streptavidin conjugated Alexa Fluor 488 (Molecular Probes, dilution: 1:200) for 2 h at room temperature.

Data analysis

To determine the amplitude of the eIPSCs and eEPSCs, ≥ 10 consecutive eIPSCs or eEPSCs were averaged and measured using pClamp 8.0 analysis software. Measurement of the amplitude and frequency of mIPSCs and mEPSCs and the exponential fit of the decaying phases of postsynaptic currents were performed for a period of 3–6 min during the control and drug responses using a peak detection program (MiniAnalysis, Synaptosoft, Leonia, NJ). Detection of events was accomplished by setting a threshold above the noise level. The cumulative probability of the amplitude and interevent interval of mEPSCs and mIPSCs was compared using the Kolmogorov-Smirnov test, which estimates the probability that two cumulative distributions are similar. The effects of drugs on the peak amplitude of eIPSCs and eEPSCs and frequency and amplitude of mIPSCs and mEPSCs were analyzed by the nonparametric (Wilcoxon

FIG. 1. Concentration-dependent effect of clonidine on evoked inhibitory postsynaptic currents (eIPSCs) in labeled paraventricular nucleus (PVN) neurons. A: original recordings of eIPSCs in a labeled PVN neuron during control and application of different concentrations of clonidine. Traces are averages of 10 consecutive responses. Stimulation artifacts are removed for clarity and indicated by arrows. B: summary data showing effect of clonidine on the peak amplitude of eIPSCs in 12 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).

FIG. 2. Effect of clonidine on evoked excitatory postsynaptic currents (eEPSCs) in labeled PVN neurons. A: raw tracings of eEPSCs in a labeled PVN neuron during control, application of different concentrations of clonidine, and CNQX (20 μM). The traces are averages of 10 consecutive responses. Stimulation artifacts are removed for clarity and indicated by arrows. B: summary data showing effect of clonidine on the peak amplitude of eEPSCs in 6 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). CLN, clonidine.
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 voltage-clamp recordings were performed on 53 PVN cells ($n = 27$ rats) labeled by FluoSpheres. The spinal cord was taken out after killing the rat and sectioned into slices (30 μm in thickness) at the level of $T_2$–$T_4$. These slices were viewed under a microscope equipped with fluorescence illumination to verify the injection and diffusion site of the tracer as documented previously (Li et al. 2003, 2004a). The injection and diffusion site of the tracer was largely located in and around the IML in the spinal cord. The labeled PVN neurons displayed a resting membrane potential ranging from $-75.3$ to $-55.4$ mV ($-61.7 \pm 6.3$ mV) and an input resistance between 468 and 634 MΩ ($526.7 \pm 42.7$ MΩ).

**Effect of clonidine on evoked IPSCs and EPSCs in labeled PVN neurons**

To examine the effect of clonidine on synaptic inputs to labeled PVN neurons, both IPSCs and EPSCs were evoked by electrical stimulation at a constant intensity. The eIPSCs were isolated at a holding potential of $-70$ mV in the presence of 20 μM CNQX, while the eEPSCs were isolated at a holding potential of 0 mV in the presence of 20 μM bicuculline (Li et al. 2003). Both eEPSCs and eIPSCs were evoked in 9 of 12 labeled PVN neurons, and only IPSCs were evoked without detectable EPSCs in the remaining 3 cells. Clonidine (5–20 μM) inhibited the peak amplitude of eIPSCs in a concentration-dependent manner ($n = 12$; Fig. 1). The peak amplitude of eIPSCs was significantly reduced by clonidine at a concentration of 5 μM, and the inhibition reached near maximum (57.5%) at a concentration of 20 μM (Fig. 1B). The effect of clonidine on eEPSCs was also examined on those nine cells with eEPSCs. Clonidine had no significant effect on the peak amplitude of eEPSCs.

**FIG. 3.** Yohimbine abolished the effect of clonidine on eIPSCs. **A:** representative recordings from a labeled PVN neuron showing application of 5 μM yohimbine blocked effect of 10 μM clonidine on eIPSCs. **B:** summary data showing that yohimbine eliminated effect of clonidine on the peak amplitude of eIPSCs in 8 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). CLN, clonidine; YHB, yohimbine.

**FIG. 4.** Inhibitory effect of clonidine on miniature IPSCs (mIPSCs) in labeled PVN neurons. **A:** raw tracings showing the mIPSCs during control, perfusion of 10 μM clonidine, and washout in a labeled PVN neuron. **B and C:** cumulative distribution plot analysis of mIPSCs of the same neuron showing distributions of the interevent interval (B) and amplitude (C) during control, application of 10 μM clonidine, and washout. **D:** superimposed averages of 100 consecutive mIPSCs obtained during control and clonidine application. Decay phase of mIPSCs was best fitted by a double-exponential function. Neither $\tau_{fast}$ (5.36 vs. 5.82 ms) nor $\tau_{slow}$ (18.91 vs. 19.53 ms) was altered during application of clonidine. **E and F:** summary data showing effect of clonidine on the frequency (E) and amplitude (F) of mIPSCs in 10 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). CLN, clonidine.
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). CLN, clonidine; PRZ, prazosin; YHB, yohimbine.

amplitude of eEPSCs at concentrations of 10 and 20 μM in six of nine neurons tested (Fig. 2, A and B). In another three cells, clonidine decreased the peak amplitude of eEPSCs from 125.3 ± 46.3 to 95.6 ± 39.1 μA (P < 0.05).

To determine the specific effect of clonidine on eIPSCs, the specific antagonist for the α2 adrenergic receptors yohimbine was used. The effective concentration of yohimbine has been determined previously (Han et al. 2002; Pan et al. 2002). In eight labeled cells tested, 5 μM yohimbine blocked the inhibitory effect of 10 μM clonidine on eIPSCs (Fig. 3). Yohimbine alone had no effect on eIPSCs of these PVN neurons in the slice preparation (data not shown).

Effect of clonidine on mIPSCs of labeled PVN neurons

To further determine the presynaptic effect of clonidine on GABAergic synaptic inputs to labeled PVN neurons, we examined the effect of clonidine on mIPSCs in 10 separate labeled PVN neurons. The mIPSCs were recorded in the presence of 1 μM TTX and 20 μM CNQX. Application of 20 μM bicusculine completely abolished mIPSCs (n = 10; Fig. 4A). Clonidine, in a concentration of 10 μM, significantly decreased the frequency of mIPSCs from 2.68 ± 0.41 to 1.22 ± 0.40 Hz (P < 0.05) without altering the amplitude and the decay time constant of mIPSCs in all 10 neurons tested (Fig. 4). The cumulative probability analysis of mIPSCs before and during clonidine application revealed that the distribution pattern of the interevent interval of mIPSCs shifted to the right in response to clonidine, while the distribution pattern of the amplitude was not changed (Fig. 4, B and C). The decay phase of mIPSCs was best fitted by a double exponential function (Fig. 4D). Neither the fast (6.85 ± 0.38 vs. 6.53 ± 0.37 ms) nor slow (22.61 ± 1.62 vs. 23.42 ± 2.24 ms) component of the decay phase of mIPSCs during clonidine application was significantly different from those during the control.

To determine if α2 adrenergic receptors mediate the effect of clonidine on mIPSCs in labeled PVN neurons, we tested the effect of 10 μM clonidine on mIPSCs before and after treatment the slices with yohimbine. The effect of clonidine on mIPSCs was abolished by 5 μM yohimbine in labeled PVN neurons (n = 9; Fig. 5, A–E). To determine whether α2 adrenergic receptors were involved in the effect of clonidine on mIPSCs, the α1 receptor antagonist, prazosin (Han et al. 2002; Pan et al. 2002), was used. Application of 20 μM prazosin had no significant effect on clonidine-induced decrease in the frequency of mIPSCs in another seven labeled PVN neurons (Fig. 5, F and G).

Effect of clonidine on paired-pulse facilitation of evoked IPSCs and EPSCs

To determine the presynaptic effect of clonidine on synaptic inputs to labeled PVN neurons, we examined the effect of 10 μM clonidine on the paired-pulse ratio (PPR) of evoked IPSCs and EPSCs. Clonidine increased the PPR (A2/A1) from 1.25 ± 0.05 to 1.61 ± 0.08 in 9 of 10 labeled PVN neurons (P < 0.05; Fig. 6, A and B). Clonidine did not change the PPR in the
remaining one neuron (1.12 vs. 1.10). On the other hand, 10 µM clonidine did not change the PPR of evoked EPSCs in seven of eight labeled neurons tested (Fig. 6C) but increased the PPR of evoked EPSCs in the remaining one neuron (1.34 vs. 1.69).

Lack of effect of clonidine on mEPSCs in labeled PVN neurons

The mEPSCs were recorded from FluoSphere-labeled PVN neurons in the presence of 1 µM TTX and 20 µM bicuculline. Neither the frequency nor the amplitude of mEPSCs was significantly affected by bath application of 20 µM clonidine in all seven additional neurons examined (Fig. 7). The effect of clonidine on mEPSCs was further analyzed by measuring the time constant of the decay phase of mEPSCs. The decay phase of mEPSCs was best fitted by a single exponential function (Fig. 7D). The decay time constant was similar during control and clonidine application (1.98 ± 0.33 vs. 2.01 ± 0.31 ms, P > 0.05).

Presynaptic location of α2A receptors in the PVN

To determine the presynaptic location of α2A receptors, double immunofluorescence labeling was performed using specific antibodies against synaptophysin and α2A receptors in the same brain section containing the PVN. All negative controls (omitting primary antibodies) displayed no detectable staining. The synaptophysin immunoreactivity in the PVN occurred mainly in the form of fine punctate deposits that often outlined neuronal cell bodies in a basket-like fashion (Fig. 8, A1 and A2). From the confocal images, numerous puncta immunoreactive for α2A receptor were present extensively in the PVN (Fig. 8, B1 and B2). The co-localization of synaptophysin and α2A receptor immunoreactivities was indicated by the color change (yellow, Fig. 8, C1 and C2). Notably, some α2A receptor immunoreactivities not co-localized with synaptophysin were also present in the PVN.

To further determine the spatial relationship between the α2A receptor, GABAAergic terminals, and the spinally projecting PVN neurons, we performed double immunofluorescence labeling of α2A receptors and GABA in brain slices in which PVN neurons were retrogradely labeled with Alexa Fluor-594 conjugated to cholera toxin B (red, Fig. 9A). This triple labeling procedure revealed that the α2A receptor (blue) was co-localized with GABA (green) immunoreactivities in the PVN (Fig. 9). Furthermore, the co-localized GABA and α2A receptor immunoreactivities were in close apposition to the labeled PVN neurons (Fig. 9).

DISCUSSION

This study examined the role of α2 adrenergic receptors in the control of excitatory and inhibitory synaptic inputs to spinally projecting PVN neurons. We found that activation of α2 receptors with clonidine significantly inhibited the evoked GABAAergic IPSCs in a concentration-dependent manner. Also, clonidine significantly increased the PPR of evoked IPSCs and reduced the frequency of mIPSCs without affecting the amplitude and decay time constant of mIPSCs. Furthermore, clonidine-induced inhibition of GABAAergic IPSCs was abolished by the α2 receptor antagonist yohimbine but not the α1 receptor antagonist prazosin. On the other hand, clonidine had little effect on glutamatergic eEPSCs or mEPSCs in most spinally projecting PVN neurons. Consistent with the electrophysiological data, presence of α2A receptors on the presynaptic GABAAergic terminals in the PVN was shown by double immunofluorescence labeling. Therefore this study provides substantial new evidence that α2 adrenergic receptors function as heteroreceptors and regulate synaptic GABA release onto PVN-spinal output neurons.

The noradrenergic synaptic inputs to the PVN neurons play an important role in the control of neuroendocrine and autonomic functions (Cunningham and Sawchenko 1988; Daftary et al. 1998; Swanson and Sawchenko 1983). Previous studies have shown that α2 adrenergic receptors and their mRNA are expressed in the PVN, although the precise location (pre- or postsynaptic sites) of α2 receptors has not been determined (Cummings and Seybold 1988; Tavares et al. 1996; Zeng and Lynch 1991). Several studies have shown that norepinephrine regulates glutamatergic and GABAAergic synaptic inputs to magnocellular or parvocellular neurons in the PVN (Daftary et al. 1998, 2000; Han et al. 2002). For instance, it has been shown that norepinephrine increases the frequency of spontaneous excitatory synaptic potential in one-third of type II (presumably parvocellular) PVN neurons (Daftary et al. 2000). In another study, norepinephrine increases spontaneous IPSCs in some parvocellular neurons but decreases spontaneous IPSCs in other parvocellular neurons in the PVN (Han et al. 2002). However, the PVN contains heterogeneous interneurons and
outputs neurons projecting to many different regions of the CNS. Thus the underlying physiological functions of these data are difficult to interpret because the magnocellular and parvocellular PVN neurons were identified solely based on the electrophysiological characters in those studies (Daftary et al. 1998, 2000; Han et al. 2002). In this study, we used retrograde labeling technique to identify the spinally projecting PVN neurons to focus on the effect of clonidine on preautonomic output neurons. The PVN-spinal neurons are considered to be an important descending pathway in the regulation of sympathetic outflow (Bains and Ferguson 1995; Pyner and Coote 2000; Ranson et al. 1998; Swanson and Sawchenko 1983). We found that clonidine produced a consistent inhibition of both the amplitude of evoked GABAergic IPSCs and the frequency of mIPSCs in all labeled neurons examined. Furthermore, clonidine increased the paired pulse ratio of evoked IPSCs and decreased the frequency of mIPSCs without altering the amplitude and the kinetics of mIPSCs, indicating that clonidine decreases the probability of GABA release through activation of $\alpha_2$ adrenergic receptors on the presynaptic nerve terminals. Therefore these data suggest that the presynaptic effect of clonidine on synaptic GABA release is mediated by $\alpha_2$, but not $\alpha_1$, adrenergic receptors. However, because highly selective antagonists for each subtype of $\alpha_2$ adrenergic receptors are not available to differentiate these subtypes, we are uncertain about the $\alpha_2$ subtypes involved in the effect of clonidine on synaptic GABA release. The $\alpha_{2A}$ subtype is predominant in the brain and thought to mediate most of the central effect of $\alpha_2$ adrenergic agonists (MacMillan et al. 1996; Tavares et al. 1996; Zeng and Lynch 1991). Furthermore, prazosin is considered to be an $\alpha_1$- and $\alpha_2$-non-A adrenergic receptor antagonist (Bylund 1995). Hence, it is possible that the presynaptic effect of clonidine on synaptic GABA release in the PVN is mediated through the $\alpha_{2A}$ subtype. It has been suggested that the norepinephrine-induced decrease in IPSCs of parvocellular neurons is mediated by the $\alpha_{2A}$ subtype (Chong et al. 2004).

Our immunocytochemistry data provide complementary new evidence that at least some $\alpha_{2A}$ adrenergic receptors are located at the presynaptic terminals in the PVN. Additionally, we
found that $\alpha_{2A}$ receptors were co-localized with the GABA immunoreactivity and were in close apposition to labeled PVN neurons, suggesting close synaptic contact of $\alpha_{2A}$-containing GABAergic terminals with PVN-spinal output neurons. Therefore this study provides strong evidence that the presynaptic $\alpha_{2A}$ receptors can function as heteroreceptors and are involved in regulation of GABAergic synaptic inputs to preautonomic PVN neurons.

In contrast to its action on GABAergic IPSCs, clonidine had little effect on the glutamatergic EPSCs in labeled PVN neurons. The reasons for this selective effect of clonidine on the GABAergic synaptic inputs are not clear. Similar to the effect of clonidine on the synaptic inputs to the spinally projecting neurons in the PVN, both NO and angiotensin II have preferential effects on GABAergic, but not glutamatergic, synaptic inputs to the PVN neurons (Li et al. 2002, 2003). Although clonidine inhibited evoked EPSCs in a few cells (3 of 9), it did not alter the amplitude of eEPSCs in the majority of labeled PVN neurons and had no effect on the frequency of mEPSCs in all the labeled PVN neurons examined. It is uncertain why clonidine reduced the amplitude of evoked EPSCs in a few neurons but had no effect on most cells tested. One possibility is that different populations of afferent terminals in the PVN were activated by electrical stimulation. It should be acknowledged that it is not possible to identify and stimulate specific afferent pathways to labeled PVN neurons in this thin slice preparation. Another possibility is that some $\alpha_2$ adrenergic receptors may be located on the soma of a few glutamatergic interneurons within the PVN. It should be noted that our study focused on the role of $\alpha_2$ adrenergic receptors in the effect of clonidine on glutamate and GABA release. Thus the potential roles of $\alpha_1$ receptors and postsynaptic $\alpha_2$ receptors in the effect of norepinephrine in the PVN were not studied. In addition to other mechanisms, the excitability of PVN neurons is regulated by both pre- and postsynaptic $\alpha_2$ receptors. Activation of postsynaptic $\alpha_2$ receptors alone (in the absence of presynaptic $\alpha_2$ receptors) may inhibit the excitability of PVN preautonomic neurons. However, the overall effect of clonidine on individual PVN neurons depends critically on the dynamic balance of its presynaptic (disinhibition) and postsynaptic (hyperpolarization) actions. Since microinjection of clonidine into the PVN increases the blood pressure by potentiation of the sympathetic outflow (Ebihara et al. 1993), the major effect of clonidine in the PVN seems to be mediated by presynaptic $\alpha_2$ receptors.

Data from this study suggest that $\alpha_2$ adrenergic receptors play an important role in regulation of GABAergic synaptic inputs to PVN preautonomic neurons. The tonic GABAergic synaptic inputs are critical in the control of the neuronal excitability of these PVN output neurons (Li et al. 2002, 2003). Based on the observation that clonidine inhibits the GABAergic synaptic inputs to spinally projecting PVN neurons, disinhibition (reduction of GABAergic input) of the PVN neurons...
by clonidine could lead to excitation of these preautonomic neurons. This possibility is supported by the in vivo study showing that norepinephrine microinjected into the PVN produces an α2 receptor-mediated increase in blood pressure in conscious rats (Harland et al. 1989). Also, this presynaptic effect of clonidine likely is the synaptic mechanism by which clonidine injected into the PVN causes a pressor response in conscious rats (Ebihara et al. 1993). However, it is important to note that we specifically examined the role of presynaptic α2 adrenergic receptors in the regulation of GABAergic and glutamatergic synaptic inputs to spinally projecting PVN neurons in this study. Nevertheless, the α2 adrenergic receptors are located on both presynaptic and postsynaptic sites. Previous studies suggest that the noradrenergic activity is increased (Almeida et al. 2000; Qualy and Westfall 1988), but the GABAergic input seems to be impaired in the hypothalamus in hypertensive rats (de Wardener 2001; Kunkler and Hwang 1995). It is likely that the presynaptic effect of clonidine on GABAergic inputs in the PVN is altered due to the reduced GABAergic inputs in hypertension. Further studies are warranted to delineate the complex interaction between the pre- and postsynaptic actions of α2 adrenergic receptors in the control of the neuronal activity of preautonomic PVN neurons in normal and pathophysiological conditions such as hypertension.

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