Microinjection of Glycine into the Hypothalamic Paraventricular Nucleus Produces Diuresis, Natriuresis, and Inhibition of Central Sympathetic Outflow

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ABSTRACT

Strychnine-sensitive glycine receptors and glycine-immunoreactive fibers are expressed in the hypothalamic paraventricular nucleus (PVN), yet the functional significance of this innervation is unclear. Therefore, these studies examined the changes in cardiovascular and renal function and renal sympathetic nerve activity (RSNA) produced by the microinjection of glycine (5 and 50 nmol) into the PVN of conscious Sprague-Dawley rats. Microinjection of glycine into, but not outside of, the PVN dose-dependently increased urine flow rate and urinary sodium excretion and decreased RSNA. At the higher dose, PVN glycine also decreased heart rate; neither 5 nor 50 nmol PVN glycine altered mean arterial pressure. The glycine (50 nmol)-evoked diuresis and natriuresis were abolished in rats continuously infused intravenously with [Arg⁸]-vasopressin. Furthermore, chronic bilateral renal denervation prevented the bradycardia

and diuresis to PVN glycine and blunted the natriuresis. In other studies, unilateral PVN pretreatment with the glycine receptor antagonist strychnine (1.6 nmol) prevented the effects of PVN glycine (50 nmol) on heart rate, RSNA, and renal excretory function. When microinjected bilaterally, PVN strychnine (1.6 nmol per site) evoked a significant increase in heart rate and RSNA without altering renal excretory function. These findings demonstrate that in conscious rats glycine acts in the PVN to enhance the renal excretion of water and sodium and decrease central sympathetic outflow to the heart and kidneys. Although endogenous PVN glycine inputs elicit a tonic control of heart rate and RSNA, the renal excretory responses to PVN glycine seem to be caused primarily by the inhibition of arginine vasopressin secretion.

Introduction

The hypothalamic paraventricular nucleus (PVN) is a key brain site involved in the central neural and humoral regulation of body fluid and electrolyte homeostasis (Badoer, 2010). Located adjacent to the third ventricle, the PVN is composed of a heterogeneous population of neurons including larger magnocellular and smaller parvocellular neurons. From the PVN, magnocellular neurons, which synthesize the neurohypophyseal hormones arginine vasopressin (AVP) and oxytocin, project to the posterior pituitary (Swanson and

Sawchenko, 1983). After synthesis/storage, AVP (and to a lesser extent, oxytocin) is transported via the hypothalamo-hypophyseal tract to the posterior pituitary and secreted into the circulation in response to an increase in plasma osmolality/sodium concentration (predominant mechanism) or a decrease in blood volume/pressure (Swanson and Sawchenko, 1983; Bisset and Chowdrey, 1988). In regard to regulation of systemic arterial blood pressure, the posterior parvocellular areas of the PVN send long descending projections to premotor sympathetic neurons in the rostral ventrolateral medulla and the nucleus of the solitary tract in the lower brainstem. Projections from the parvocellular neurons of the PVN also synapse directly onto sympathetic preganglionic motoneurons of the intermediolateral cell column of the thoracolumbar spinal cord. Finally, neurosecretory parvocellular neurons of the PVN also project to the external layer of the median eminence where AVP and corticotropin-releasing hormone are secreted into portal blood to coordinate hypothalamic-pituitary-adrenal system activity (Badoer, 2010).

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ABBREVIATIONS: PVN, paraventricular nucleus; SON, supraoptic nucleus; RSNA, renal sympathetic nerve activity; AVP, arginine vasopressin; aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; C, control.

Circulating blood levels of AVP are determined largely by the electrical activity of the magnocellular neurons in the PVN and supraoptic nucleus (SON) (Badoer, 2010). In the PVN (and SON), membrane-bound voltage-gated potassium and calcium currents contribute to the phasic activity of AVP magnocellular neurons. Magnocellular activity is also tightly regulated by neurotransmitters and neuromodulators through either postsynaptic mechanisms or by presynaptic modulation, largely of excitatory glutamatergic and inhibitory GABAergic synaptic inputs (Badoer, 2010).

In addition to GABA, glycine is a major inhibitory neurotransmitter in the brainstem and medulla (Hernandes and Troncone, 2009; Keck and White, 2009). After stimulation, strychnine-sensitive glycine receptors play an important inhibitory role in neurotransmission through gating of chloride channels. In addition to their abundance in the spinal cord and brainstem, glycine receptor mRNAs and protein are expressed in forebrain structures including the PVN (Hernandes and Troncone, 2009). In the PVN, the parvocellular region receives a dense innervation of glycine-immunoreactive fibers, whereas a smaller quantity of these fibers is expressed in the magnocellular division of this nucleus (Rampton et al., 1996). These findings suggest that in addition to the well established involvement of glycine as an inhibitory neurotransmitter in the spinal cord and lower brainstem, glycine and its receptor(s) may have a role in neurotransmission in higher brain structures. In this regard, intracerebroventricular administration of glycine to rats has been reported to dose-dependently decrease heart rate and blood pressure and enhance epinephrine-induced reflex bradycardia (Yang and Lin, 1983). However, it remains unknown whether glycine receptor pathways in the PVN mediate centrally evoked changes in systemic cardiovascular and/or renal function and, if so, whether glycine-induced alterations in neural and/or humoral pathways are involved.

Therefore, these studies investigated whether glycine pathways in the PVN may have a physiological role in the central control of cardiovascular and renal function. We first determined the cardiovascular and renal excretory responses produced by microinjection of glycine into the PVN of conscious rats. Studies were then performed to examine the roles of AVP and intact renal nerves in mediating the cardiovascular and renal responses to PVN glycine. Participation of the renal sympathetic nerves in mediating glycine-induced changes in renal excretory function was examined by directly measuring changes in renal sympathetic nerve activity (RSNA) and repeating the experimental protocol in rats that had undergone chronic bilateral renal denervation. Finally, separate experiments were performed to determine the effects produced by bilateral microinjection of strychnine, a highly selective and potent competitive glycine receptor antagonist (Lee et al., 2009), into the PVN on cardiovascular and renal excretory function and RSNA. For these studies, strychnine was injected into animals alone or as a pretreatment before glycine injection. Strychnine was used to pharmacologically block a potential ongoing tonic influence of glycine in the PVN on a given cardiovascular or renal excretory parameter and to test the receptor selectivity of the action of glycine in this brain nucleus.

Materials and Methods

Animals. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 285 to 400 g were housed in a controlled environment and allowed free access to laboratory rat chow and water. All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Louisiana State University Health Sciences Center Animal Care and Use Committee.

Surgery. Aseptic technique was used for all surgeries. Five to 7 days before investigation, rats were stereotactically implanted with a chronic stainless-steel cannula into the PVN for drug/vehicle injection into this nucleus. On the day of surgery, rats were anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). A 2-cm incision was made to expose the scalp, and connective tissue was removed to expose the cranial sutures. A 26-gauge stainless-steel cannula was directed at the dorsal parvocellular subdivision of the PVN at a 10° angle from the vertical through a burr hole located stereotactically 1.7 mm posterior to the bregma, 1.95 mm laterally to the midline, and 7.8 mm ventral from the skull surface. Inner stylets were inserted into the guide cannulae to prevent their obstruction by clots. The guide cannulae were secured to the skull with three stainless-steel machine screws and dental acrylic. All rats received ampicillin (50 mg/kg s.c.) to prevent postsurgery infection. The animals were then placed in individual plastic cages and allowed to recover from the stereotaxic surgery for at least 5 to 7 days before their use in an experimental protocol.

On the morning of the experimental day, rats were anesthetized with methohexital sodium (Brevital; 20 mg/kg i.p., supplemented with 10 mg/kg as needed; Eli Lilly & Co., Indianapolis, IN), and polyethylene catheters (PE-50; BD Diagnostics, Sparks, MD) were implanted into the left femoral artery and vein for recording of arterial blood pressure and infusion of isotonic saline or AVP, respectively. Through a suprapubic incision, a flanged polyethylene cannula (PE-240; BD Diagnostics) was then inserted into the urinary bladder. The bladder catheter was then exteriorized and secured by suturing to adjacent muscle, tissue, and skin.

For certain experiments, rats (still anesthetized with Brevital) were also implanted with a renal nerve recording electrode for direct measurement of RSNA. For these studies, the left kidney was exposed via a retroperitoneal approach and a renal nerve bundle was dissected carefully and freed from the surrounding tissue under a dissecting microscope ($\times 25$). The nerve was placed on a bipolar electrode made of Teflon-coated platinum wire (Cooner Wire, Chatsworth, CA). Spike potentials were amplified ($\times 10,000$ – $50,000$) and filtered (low, 30 Hz; high, 3000 Hz) with a Grass P511 Bandpass Amplifier (Grass Instruments, Quincy, MA). The amplified and filtered signal was channeled to a Grass model 7DA polygraph for visual evaluation, an audio amplifier-loudspeaker (Grass model AM 8 Audio Monitor) for auditory evaluation, and a rectifying voltage integrator (Grass model 7P10) and continuously recorded on the Grass polygraph and using a data acquisition system (model MP100; Biopac Systems, Inc., Santa Barbara, CA). The quality of the renal sympathetic nerve signal was assessed by its pulse synchronous rhythmicity. When an optimal renal sympathetic nerve activity signal was observed, the recording electrode was fixed to the renal nerve branch with and covered with a silicone-based impression material (President light body; Coltene AG, Alstatten, Switzerland). The electrode cable was then secured in position by suturing it to the abdominal trunk muscles. Finally, the electrode cable was exteriorized, and the flank incision was closed in layers.

After surgical preparation rats were placed in a rat holder (a chamber of stainless-steel rods connected by Plexiglas ends; the metal rods formed an inverted U shape and a flat base in which the rat would sit) to minimize movement and damage to the renal nerve recording electrode preparation and to permit steady-state urine collection. The animals were then allowed 4 to 6 h to fully

recover from anesthesia during which time an infusion (50 μ l/min i.v.) of isotonic saline was started and continued for the duration of the experiment. Before the start of the experiment, the arterial catheter was flushed and attached to a pressure transducer (model P23XL, Viggo-Spectramed, Oxnard, CA), and the urinary bladder catheter was led to a collection vial. Heart rate was derived from the pulse pressure by a tachograph (Grass model 7 P4H), and mean arterial pressure, heart rate, and RSNA were recorded on a Grass model 7 polygraph and a Biopac data acquisition system (model MP100 and AcqKnowledge 3.7.2 software; Biopac Systems, Inc.).

Experimental Protocols. Studies were performed to examine changes in the cardiovascular, renal excretory, and RSNA responses to unilateral (left) microinjection of glycine into the PVN of conscious rats. After stabilization of cardiovascular, renal excretory, and RSNA parameters, urine was collected during a 20-min control period. Next, a 33-gauge injection needle connected to 10- μ l micro-syringe was inserted into the guide cannula, so that the tip of the injection needle extended beyond the guide cannula by 0.5 mm and reached the PVN. Vehicle or glycine (5 or 50 nmol) dissolved in the vehicle was injected into the PVN at a volume of 60 nl over 10 s. Artificial cerebrospinal fluid (aCSF) of the following composition was used as vehicle: 140 mM NaCl, 3.35 mM KCl, 1.15 mM $MgCl_2$, 1.26 mM $CaCl_2$, 1.2 mM Na_2HPO_4 , and 0.3 mM NaH_2PO_4 , pH 7.4. A 30-s period was allowed for diffusion. Then, the injection cannula was withdrawn. Immediately after PVN injection, urine was collected, and changes in cardiovascular and RSNA were recorded during eight consecutive 10-min experimental periods.

Separate experiments were performed to determine whether changes in circulating levels of AVP play a role in mediating the cardiovascular and/or renal excretory responses produced by the microinjection of glycine into the PVN. For these studies, the experimental protocol described above was repeated in rats ($n = 5$; intact renal nerves) with the exception that after recovery from surgical preparation AVP was added to the isotonic saline vehicle and continuously infused intravenously throughout the PVN glycine microinjection experiment at a rate of 85 pg/kg/min.

The role of intact renal nerves in mediating the cardiovascular and renal excretory responses produced by the injection of glycine into the PVN was examined by repeating the PVN glycine microinjection protocol in six rats having undergone chronic bilateral renal denervation 10 to 13 days before experimentation. Renal denervation was performed via bilateral flank incisions and stripping the renal arteries and veins of adventitia, cutting the renal nerve bundles, and coating the vessels with a solution of 10% phenol in absolute ethanol as described previously (DiBona and Sawin, 1983). This renal denervation procedure prevents the renal vasoconstrictor response to suprarrenal lumbar sympathetic nerve stimulation, prevents the antinatriuretic response to environmental stress, reduces renal catecholamine histofluorescence to undetectable levels, and reduces renal tissue noradrenaline concentration to <5% of control for up to 15 days postdenervation (DiBona and Sawin, 1983). Because we have previously and repeatedly verified that this renal denervation procedure completely removes the influence of the renal nerves on kidney function (Kapusta and Kenigs, 1999; Krowicki and Kapusta, 2006), verification of renal denervation was not performed in these studies. Five to 8 days after bilateral renal denervation, the same rats were anesthetized with ketamine/xylazine and implanted with a chronic PVN cannula as described previously for microinjection of glycine into this brain site on the day of study.

Additional experiments were performed to evaluate the receptor selectivity of glycine in the PVN and determine whether the native glycine peptide and receptor system in the PVN plays a tonic role in the regulation of cardiovascular and renal function and RSNA. For these studies the selective glycine receptor antagonist strychnine (Lee et al., 2009) was microinjected into the PVN (1.6 nmol) 10 min before vehicle or glycine (50 nmol). Changes in cardiovascular and renal excretory function and RSNA were measured during consecu-

tive 10-min periods before (control) and after (experimental) drug injection for 80 min.

Histology. At the end of all microinjection experiments, the site of drug injection in the brain was examined functionally and histologically. The microinjection of L-glutamate (15 nmol) was used to confirm localization of the tip of an injection needle in the PVN. Typical responses to L-glutamate in the PVN included increases in heart rate and mean arterial pressure (Krowicki and Kapusta, 2006). Next, the injection site was marked by the injection of 1% pontamine sky blue dye in the same manner as glycine injection. The animals were euthanized with an overdose of methohexital sodium and perfused transcardially with saline and 4% paraformaldehyde. The brains were removed, postfixed in the same solution, sectioned at 50 μ m, and counterstained with neutral red dye for histological confirmation of injection sites. Injections were considered acceptable when the cannula tip was within a distance of 0.5 mm of the PVN boundaries (Krowicki and Kapusta, 2006).

Analytical Techniques. Urine volume was determined gravimetrically. Urine sodium concentration was measured by flame photometry (model 943; Instrumentation Laboratories, Lexington, MA) and urine osmolality by a vapor pressure osmometer (model 5500; WESCOR, Logan, UT). Free water clearance was calculated as a difference between the rate of urine volume (ml) per minute and the osmolar clearance. Integrated RSNA is expressed as microvolt-seconds per 1-s intervals. For each 10-min control and experimental period, the values for integrated RSNA were sampled over the entire collection period, and the numbers were averaged. Because of the limitations of comparing values for multifiber RSNA between animals, data for RSNA were expressed as the percentage of the control, with the baseline control values for each animal taken as 100%. After completion of each experiment, the level of postmortem background noise was measured, and the value was then subtracted from all control and experimental values of RSNA.

Drugs. Glycine sodium salt, strychnine hydrochloride, and L-glutamate monosodium were purchased from Sigma (St. Louis, MO). All solutions were freshly prepared in aCSF, pH 7.4. Doses of glycine and strychnine used were based on previous studies by Pimentel et al. (2003) and Ferreira et al. (2001).

Statistical Analysis. Results are expressed as the mean $\pm$ S.E.M. The magnitude of changes in cardiovascular, renal excretory, and RSNA parameters at different time points after injection of drugs in the PVN were compared with respective group control values by a one-way repeated-measures analysis of variance (ANOVA) with a subsequent Dunnett's multiple comparison test. Differences occurring between treatment groups were assessed by a two-way repeated measures (mixed model) ANOVA with treatment being one fixed effect and time another, with the interaction included. The time (minutes) was the repeated factor, and post hoc analysis was performed using the Bonferroni's post-test. Data were verified for normality of distribution and equality of variances. If needed, data were normalized using a log10 transformation for the purpose of statistical analyses; however, results are reported in standard units. Statistical testing was carried out using Prism software (GraphPad Software, Inc., San Diego, CA) and SigmaStat programs (Systat Software, Inc., San Jose, CA). In each case, statistical significance was defined as $p < 0.05$.

Results

Cardiovascular and Renal Effects of Glycine Microinjected into the PVN. Figure 1 illustrates the cardiovascular, renal excretory, and RSNA responses produced by unilateral microinjection of glycine (5 or 50 nmol) or aCSF (vehicle) into the PVN of conscious Sprague-Dawley rats. After microinjection of glycine at the lower dose of 5 nmol, RSNA decreased, whereas mean arterial pressure and heart rate did not change. The glycine-induced reduction in RSNA

Fig. 1. Cardiovascular and renal effects of glycine in the PVN of intact rats. The values are mean $\pm$ S.E.M. and illustrate the systemic cardiovascular, renal sympathetic nerve, and renal excretory responses produced by the microinjection of 5 nmol glycine (Δ , $n = 5$), 50 nmol glycine ($\circ$, $n = 7$), or aCSF (vehicle; $\square$, $n = 7$) into the PVN of rats. Urine samples were collected during control (C, baseline 20-min time period) and immediately after drug/vehicle microinjection for 80 min, denoted as time periods 10 to 80 min (consecutive 10-min samples). MAP, mean arterial pressure; HR, heart rate; V, urine flow rate; U_{NaV} , urinary sodium excretion. # and *, statistically significant ($p < 0.05$) for glycine at doses of 5 and 50 nmol, respectively, compared with the respective group time C value.

was rapid in onset and significantly different from predrug control levels (C) from 40 to 70 min after injection, with a nadir at the 40-min time point (C: 100%; 40 min: $71 \pm 5\%$, $p < 0.05$). The 5-nmol dose of glycine also produced a significant increase in urine flow rate and urinary sodium excretion. The peak diuretic response to 5 nmol glycine occurred 50 min after PVN drug injection (C: $50 \pm 6 \mu\text{l/min}$; 50 min: $96 \pm 16 \mu\text{l/min}$, $p < 0.05$). Microinjection of 5 nmol of glycine into the PVN evoked an increase in urinary sodium excretion with the peak natriuresis observed at the 30-min time point (C: $7.6 \pm 0.7 \mu\text{eq/min}$; 30 min: $111.1 \pm 1.1 \mu\text{eq/min}$, $p < 0.05$). When microinjected at the higher dose of 50 nmol, glycine produced a significant decrease in RSNA that was sustained throughout the experimental protocol [time points 10–80 min (nadir 20 min: $69 \pm 3\%$ of C, $p < 0.05$)]. The 50-nmol dose of glycine also decreased heart rate (time points 10 and 20 min) with the peak bradycardia occurring at the 10-min time point (C: $349 \pm 8 \text{ bpm}$; 10 min: $344 \pm 8 \text{ bpm}$, $p < 0.05$) without altering mean arterial pressure. At the 50-nmol dose, glycine also significantly increased urine flow rate 40 to 70 min after injection, with the peak diuresis observed 50 min after drug injection (C, $37 \pm 4 \mu\text{l/min}$; 50 min: $138 \pm 18 \mu\text{l/min}$, $p < 0.05$). The magnitude of diuresis produced by the

5- and 50-pmol doses of glycine was significantly different ($p < 0.05$) at time points 50 and 60 min. Compared with the predrug control period (C), microinjection of 50 nmol of glycine into the PVN evoked a marked increase in urinary sodium excretion with the peak natriuresis observed at the 30-min time point (C: $7.2 \pm 0.5 \mu\text{eq/min}$; 30 min: $15.6 \pm 1.4 \mu\text{eq/min}$, $p < 0.05$). The magnitude of urinary sodium excretion produced by the 5- and 50-pmol doses of glycine at the 30-min time point was significantly different ($p < 0.05$, two-way ANOVA). Microinjection of aCSF (vehicle) into the PVN ($n = 7$) did not significantly alter any cardiovascular, renal excretory, or RSNA parameter over the course of the experiment (Fig. 1).

Figure 2 depicts the changes in free water clearance produced by the microinjection of glycine (5 and 50 nmol) or aCSF into the PVN for the same rats in which data are depicted in Fig. 1. At the lower dose (5 nmol), glycine evoked a delayed, but significant, increase in free water clearance 60 min after drug injection (C: $-64.2 \pm 12.6 \text{ ml/min}$; 60 min: $19.0 \pm 15.5 \text{ ml/min}$, $p < 0.05$). Likewise, when microinjected into the PVN at a higher dose of 50 nmol glycine evoked significant increases in free water clearance at the 50- and 60-min time points with a peak at 60 min (C: -77.1 ± 14.8

Fig. 2. Changes in free water clearance (C_{H_2O}) produced by PVN glycine at a dose of 5 nmol (Δ , $n = 5$) or 50 nmol ($\circ$, $n = 7$) or aCSF ($\square$, $n = 7$) in the same rats for which cardiovascular and renal excretory data are depicted in Fig. 1. Values are mean $\pm$ S.E.M. # and *, statistically significant ($p < 0.05$) for glycine at a dose of 5 and 50 nmol, respectively, compared with the respective group time C value.

ml/min; peak 60 min: 21.1 ± 13.8 ml/min, $p < 0.05$). Microinjection of aCSF did not significantly alter free water clearance over the course of the study.

The histological identified sites into which glycine (5 and 50 nmol) were microinjected into the PVN of animals for which data are shown in Fig. 1 are illustrated in Fig. 3. Injections were considered acceptable if the cannula tip was within a distance of 0.5 mm of the PVN boundaries (Krowicki and Kapusta, 2006). Several sites below and/or lateral to the PVN served as anatomical controls for the glycine injections. No significant responses were elicited from these microinjections (data not shown).

Cardiovascular and Renal Effects of Glycine Microinjected into the PVN of Rats Infused with AVP. The cardiovascular and renal responses produced by the microinjection of glycine (50 nmol) or aCSF into the PVN of conscious rats continuously infused intravenously with isotonic saline containing AVP (85 pg/kg/min; $n = 5$) are shown in Fig. 4. In these studies it was observed that the continuous infusion of AVP during the first 40 min before the start of the experimental protocol (i.e., stabilization period) produced a slight, but not statistically significant, increase in mean arterial pressure, which corresponded with a significant decrease in basal levels for heart rate and RSNA (data not shown). In this and previous studies (Krowicki and Kapusta, 2006), the AVP-induced decrease in heart rate tended to reach a plateau level in the first 30 min and remained constant thereafter throughout the subsequent AVP infusion protocol (i.e., 80 min after aCSF injection). As shown in Fig. 4, the diuretic

Fig. 3. Schematic representations of serial coronal sections from the rostral (-1.4 mm) to the caudal (-2.12 mm) extent of the region of the PVN. Coordinates are in reference to bregma using standard sections from the atlas of Paxinos and Watson (1986). Open triangles (glycine 5 nmol) and open circles (glycine 50 nmol), shown in the vicinity of left PVN, represent sites of termination of injections considered to be within the PVN boundaries. Open squares, shown in the vicinity of right PVN for clarity, illustrate sites of injection of aCSF, whereas shaded circles represent termination sites outside the PVN at which glycine (50 nmol) failed to elicit responses. AH, anterior hypothalamic area, f, fornix; 3V, third ventricle.

and natriuretic responses produced by the microinjection of glycine into the PVN of conscious rats (see Fig. 1) were completely prevented in rats infused intravenously with AVP. In contrast, intravenous AVP infusion did not alter the ability of PVN glycine to decrease heart rate or RSNA. In AVP-infused rats, PVN glycine produced a significant decrease in RSNA (nadir 40 min; $55 \pm 6\%$ of C), which returned to predrug control levels 50 min after drug injection. Microinjection of aCSF did not significantly alter any cardiovascular or renal parameter over the course of the study.

Cardiovascular and Renal Effects of Glycine Microinjected into the PVN of Renal-Denervated Rats. Injection of glycine (50 nmol) into the PVN failed to alter mean arterial pressure or heart rate in chronic bilateral renal

Fig. 4. Cardiovascular and renal effects of glycine in the PVN of rats infused intravenously with AVP. Values are means $\pm$ S.E.M. and illustrate the systemic cardiovascular, renal sympathetic nerve, and renal excretory responses produced by the microinjection of glycine into the PVN of conscious rats continuously infused intravenously with AVP (85 pg/kg/min; $\bullet$, $n = 5$) compared with responses to PVN aCSF in rats infused intravenously with AVP (85 pg/kg/min; $\circ$, $n = 5$). Thirty minutes after the start of AVP infusion, urine samples were collected during control (C, 20-min time period) and immediately after the microinjection of glycine for 80 min, denoted as time periods 10 to 80 min (consecutive 10-min samples). Abbreviations are the same as in Fig. 1. *, statistically significant ($p < 0.05$) for glycine in rats infused intravenously with AVP compared with the respective group time C value.

denervated rats. In renal denervated rats, baseline levels for urine flow rate and urinary sodium excretion were elevated compared with control values in intact rats. In comparison with the marked responses observed in rats with an intact kidney innervation (as shown in Fig. 1), PVN glycine did not produce marked diuretic and natriuretic responses in renal denervated animals. However, after injection of glycine into the PVN (i.e., 10-min time point) of renal denervated rats, urine flow rate was decreased at 10 and 20 min with a nadir at 20 min (C, $59 \pm 9 \mu\text{l/min}$; 20 min: $24 \pm 6 \mu\text{l/min}$, $p < 0.05$; $n = 6$). Likewise, urinary sodium excretion was initially decreased at the 10-min time point (C: $9.6 \pm 0.7 \mu\text{eq/min}$; 10 min: $5.1 \pm 1.1 \mu\text{eq/min}$, $p < 0.05$; $n = 6$).

Cardiovascular and Renal Responses Produced by PVN Glycine in Conscious Rats Pretreated with Strychnine. Figure 5 illustrates the peak changes in cardiovascular and renal responses produced by the unilateral microinjection of aCSF or glycine (50 pmol) into the PVN of conscious rats ($n = 5/\text{group}$) pretreated (10 min) in the same brain site with the selective glycine receptor antagonist strychnine (1.6 nmol). Unilateral microinjection of strychnine with subsequent aCSF treatment evoked a significant increase in RSNA from 10- to 60-min time points with a peak at 20 min (peak: $123 \pm 12\%$ of C, $p < 0.05$); Systemic hemodynamic or renal excretory function was not altered. In separate animals, this dose of strychnine completely prevented

the marked renal sympathoinhibitory, diuretic, and natriuretic excretion typically produced by PVN glycine. In addition, the bradycardic response typically produced by PVN glycine injection in naive animals (Fig. 1) was converted to a tachycardia in animals that received strychnine pretreatment (C: 380 ± 14 bpm; peak 20 min: 410 ± 22 bpm, $p < 0.05$).

Cardiovascular and Renal Responses Produced by Bilateral Blockade of Glycine Receptors in the PVN of Conscious Rats. As shown in Fig. 6, the bilateral microinjection of strychnine (1.6 nmol per site) into the PVN of conscious rats produced a slight, but statistically significant, decrease in mean arterial pressure 70 min after treatment (C: 142 ± 3 mm Hg; nadir 70 min: 135 ± 4 , $p < 0.05$). Strychnine also evoked a significant increase in heart rate (C: 347 ± 7 bpm; peak 10 min: 375 ± 8 bpm, $p < 0.05$) and RSNA after bilateral PVN microinjection (C: 100%; peak 40 min: $131 \pm 5\%$, $P < 0.05$). No significant changes in renal excretory function were observed in these animals. Bilateral microinjection of aCSF into the PVN did not significantly alter cardiovascular or renal function over the course of the study.

Discussion

The findings of these studies demonstrate that microinjection of glycine into the PVN produces bradycardia, renal sympathoinhibition, and dose-dependent increases in water

Fig. 5. Cardiovascular and renal effects produced by PVN glycine microinjection in rats pretreated with a glycine receptor antagonist. Values are means $\pm$ S.E.M. and illustrate the cardiovascular, renal sympathetic nerve, and renal excretory responses produced by microinjection of glycine (50 nmol) or aCSF into the right PVN of rats that received previous (10 min) PVN pretreatment (ipsilateral) with the selective glycine receptor antagonist strychnine (1.6 nmol). A 20-min urine sample was collected during control (C, 20-min time period). Then strychnine was injected into the PVN and allowed 10 min to distribute. Glycine ($\blacklozenge$, $n = 5$) or aCSF ($\diamond$, $n = 5$) was then microinjected into the PVN of each group, and urine samples were immediately collected for 80 min, denoted as time periods 10 to 80 min (consecutive 10-min samples). Abbreviations are the same as in Fig. 1. * and #, statistically significant ($p < 0.05$) for PVN aCSF and glycine in rats pretreated in the PVN with strychnine, respectively, compared with the respective group time C value.

and sodium excretion from the kidneys of conscious rats. Marked increases in urine output and free-water clearance in response to glycine in the PVN suggest that this neurotransmitter may have an inhibitory influence on magnocellular neuronal activity and the release of AVP into the systemic circulation. This possibility is also suggested by the observation that taurine, which has affinity for glycine receptors, has an inhibitory influence on rat SON magnocellular neurons *in vitro* (Hussy et al., 2000) and *in vivo* (Huang et al., 2006). In accordance with this premise, we demonstrated that low-dose intravenous AVP infusion prevented the diuretic (and natriuretic) response to microinjection of glycine into the PVN. Although plasma levels of AVP were not measured in the present study, these results provide strong evidence that inhibition of AVP secretion contributes to the diuretic and natriuretic responses to PVN glycine. As noted above, glycine is not the only ligand for glycine receptors. The endogenous amino acids β -alanine and taurine have also been identified in the hypothalamus (Choi et al., 2000) and are able to activate extrasynaptic glycine receptors (Albrecht and Schousboe, 2005). However, further studies are required to determine whether PVN alanine and/or taurine are able to enhance the renal excretion of water in rats similar to that elicited by PVN glycine.

In addition to diuresis, activation of glycine receptors in the PVN produced a robust natriuresis. This change was accompanied by a significant and long-lasting reduction in RSNA. Decreases in RSNA are known to produce reciprocal increases in urinary sodium and water excretion by decreasing the renal sodium and water reabsorption throughout the nephron (DiBona, 2001). To determine whether the PVN glycine-induced natriuresis was mediated by a pathway involving intact renal nerves, we examined the renal excretory responses to glycine in chronic bilaterally renal denervated rats. In renal denervated rats, glycine did not produce a marked increase in urinary sodium excretion, therefore suggesting that intact renal nerves seem to be required to mediate the natriuresis to PVN glycine. However, it is important to note that in these renal denervated animals the natriuresis to PVN glycine may have, at least in part, been masked by a prior reduction in urine output and urinary sodium excretion (marked) that occurred immediately after PVN microinjection of glycine. The possibility that the renal nerves may instead only have a contributory role in mediating the renal excretory responses to PVN glycine is supported by the observation that diuretic and natriuretic, but not renal sympathoinhibitory, responses to PVN glycine were completely abolished in rats infused with low doses of AVP.

Fig. 6. Changes in cardiovascular and renal function produced by bilateral glycine receptor blockade with strychnine (1.6 nmol per site) in the PVN of conscious rats. Values are means $\pm$ S.E.M. and illustrate the cardiovascular, renal sympathetic nerve, and renal excretory responses produced by the bilateral microinjection of strychnine (1.6 nmol/site; $\bullet$, $n = 5$) or aCSF ($\circ$, $n = 5$) into the PVN of conscious rats. Urine samples were collected during control (C, 20-min time period) and immediately after microinjections of strychnine or aCSF for 80 min, denoted as time periods 10 to 80 min (consecutive 10-min samples). Abbreviations are the same as in Fig. 1. *, statistically significant ($p < 0.05$) compared with the respective group time C value.

Our findings demonstrate that the diuretic and natriuretic responses to PVN glycine were abolished by PVN pretreatment of animals with strychnine, thus demonstrating the role of glycine receptors in these renal excretory responses. However, instead of simply blocking the inhibitory responses on heart rate and RSNA, we observed that glycine produced tachycardia and renal sympathoexcitation in strychnine-treated animals. This observation suggests that in this setting (i.e., blockade of glycine receptors), the cardiovascular and renal sympathetic effects of glycine in the PVN may be mediated through strychnine-insensitive glycine receptors, which are allosteric binding sites in the *N*-methyl-D-aspartate receptor-channel complex (Furuya and Ogura, 1997). This is in line with current reports indicating that glycine can also act as a stimulatory transmitter in forebrain areas (Hernandes and Troncone, 2009).

As suggested above, the findings of this study suggest that endogenous activation of glycine pathways in the PVN may contribute to the renal handling of water and osmoregulation. In addition, it may be speculated that PVN glycine may have an important physiological role in regulating central sympathetic outflow to the heart. In support of this premise,

endogenous glycine levels have been shown to be increased in the micordialysate from the PVN during a time in which arterial blood pressure is increased via intravenous phenylephrine (Yin et al., 2009). In these studies, microinjection of strychnine into the PVN enhanced the pressor response to intravenous phenylephrine and attenuated the baroreflex-induced bradycardia. Furthermore, and in agreement with the findings of the present study, perfusion of glycine into the PVN enhanced the baroreflex-induced bradycardia, but not pressor response to intravenous phenylephrine (Yin et al., 2009).

We also provide direct evidence that the endogenous glycinergic system in the rat PVN is tonically active. The evidence is based on our observations that bilateral microinjection of the glycine receptor antagonist strychnine into the PVN of conscious rats produced tachycardia and a marked increase in RSNA. These are responses that are opposite in direction to the effects of PVN glycine in naive animals. In contrast, however, it seems that endogenous PVN glycine pathways are not tonically involved in the control of AVP secretion. This is suggested by the observation that bilateral PVN microinjection of strychnine did not alter urine flow

rate or urinary sodium excretion. Considering that bilateral strychnine did increase RSNA, it could be predicted that PVN glycine receptor blockade may decrease urinary sodium excretion. Although this was not the case, this may potentially be explained by activation of compensatory mechanisms (e.g., changes in renal vascular tone and renal hemodynamics) that offset the influence of enhanced renal sympathetic tone on the renal tubular reabsorption of sodium.

Together, the findings of these investigations demonstrate that the activation of glycine receptor pathways in the PVN of conscious rats evokes significant changes in heart rate (bradycardia) and the renal excretion of water and sodium. The diuretic and natriuretic responses to PVN glycine occur through pathways that concurrently involve neural (renal sympathoinhibitory) and humoral (AVP inhibitory; predominant) influences on the renal excretion of water and sodium. In addition, this study provides evidence for a physiological role for the endogenous glycine system in the PVN in the tonic inhibitory control of central sympathetic outflow to the heart and kidneys. On the basis of these findings, it may be speculated that the endogenous glycine system in the PVN may participate in the inhibitory control of AVP secretion and RSNA under stressful/pathological conditions in which it is necessary to promote the renal excretion of water and sodium to maintain body fluid/electrolyte homeostasis. In addition, endogenous glycine may act in the PVN via strychnine-sensitive glycine receptors to provide an inhibitory influence on central sympathetic outflow to the heart, which may potentially facilitate the cardiac baroreflex. Further studies are required to fully understand the importance of this novel system and determine how glycine interacts with other neurotransmitters in the PVN in the control of cardiovascular and renal function.

Authorship Contributions

Participated in research design: Krowicki and Kapusta.

Conducted experiments: Krowicki.

Contributed new reagents or analytic tools: Kapusta.

Performed data analysis: Krowicki.

Wrote or contributed to the writing of the manuscript: Krowicki and Kapusta.

Other: Kapusta acquired funding for the research.

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