Phosphate-Activated Glutaminase-Containing Neurons in the Rat Paraventricular Nucleus Express Angiotensin Type 1 Receptors

Nan Jiang, Peng Shi, Hongwei Li, Shun Lu, Leah Braseth, Adolfo E. Cuadra, Mohan K. Raizada, Colin Sumners

Abstract—The centrally mediated cardiovascular regulatory actions of angiotensin II in normal and hypertensive rats include angiotensin II type 1 receptor (AT1R)–mediated actions at the paraventricular nucleus (PVN) of the hypothalamus. Because the PVN consists of multiple neuronal populations, it is important to understand which neuronal types in the PVN are influenced by angiotensin II. Here we have developed a viral vector (Adeno-associated vector 2 [AAV2]-PAG-eGFP [PAG; phosphate-activated glutaminase promoter]) to drive expression of green fluorescent protein (GFP) primarily within glutamate neurons. At 10 to 14 days after bilateral microinjection (200 nL per side; 1.2×10¹² genome copies) of AAV2-PAG-eGFP into adult Sprague-Dawley rat PVN, animals were euthanized and brains removed and used for isolation and culture of PVN neurons. Fluorescence microscopy and immunostaining using neuron and PAG-specific antibodies revealed the presence of GFP-containing glutamatergic neurons in these PVN cultures. Whole-cell patch-clamp recordings demonstrated that angiotensin II (100 nmol/L) produced a 16% decrease in delayed rectifier potassium current in ~50% of the GFP-containing neurons, an effect that was abolished by the AT1R antagonist losartan (1 μmol/L). Consistently, 9 of 28 GFP/PAG-expressing neurons contained AT1R mRNA, as indicated by single-cell RT-PCR. Furthermore, specific GFP/PAG-positive neurons in the PVN that project to the rostral ventrolateral medulla of the brain stem express immunoreactive AT1R. In conclusion, we have demonstrated the presence of functional AT1R on PAG-positive (largely glutamate) neurons within rat PVN, certain of which project to the rostral ventrolateral medulla. (Hypertension. 2009;54:00-00.)

Key Words: angiotensin II ■ paraventricular nucleus ■ phosphate-activated glutaminase ■ potassium current ■ AT1 receptor

The paraventricular nucleus (PVN) of the hypothalamus has a key role in the regulation of sympathetic outflow, neuroendocrine secretions, and behavioral homeostatic responses. Activation of PVN neurons contributes to increases in sympathetic tone associated with conditions such as hypertension, water deprivation, increased plasma osmolality, and heart failure. The PVN contains heterogeneous populations of neurons that can be differentiated on the basis of morphological, phenotypic, or electrophysiological criteria. For example, the PVN contains glutamate, oxytocin (OT), vasopressin (AVP), corticotropin-releasing hormone (CRH), enkephalin (ENK), and a small number of γ-aminobutyric acid (GABA) neurons. In general, from a morphological standpoint, the PVN can be separated into large magnocellular neurons and smaller parvocellular neurons. The magnocellular neurons synthesize and secrete OT or AVP into the circulation from terminals at the posterior pituitary. The smaller parvocellular neurons, which include populations of most of the other transmitters, as well as AVP and OT neurons, have different projection sites. Some parvocellular neurons influence sympathetic outflow by projecting to brain stem cardiovascular regulatory sites, such as the rostral ventrolateral medulla (RVLM), or directly to preganglionic sympathetic neurons in the intermediolateral column of the spinal cord. Other parvocellular neurons (CRH) project to the median eminence and influence cardiovascular regulation, particularly during stress. Magnocellular and parvocellular neurons can also be distinguished by different thresholds of action potential and A-type K⁺ current properties.

Because of its central role in the regulation of sympathetic outflow, it is important to understand the factors that modulate the activity of PVN neurons. One such factor is angiotensin II (Ang II), which acts in the brain via its type 1 receptors (AT1Rs) to increase sympathetic outflow and blood pressure, and the high densities of AT1R present in the PVN mediate the pressor action of centrally injected Ang II.
Overactivity of central nervous system (CNS) AT1Rs, including those in the PVN, contributes to neurogenic hypertension. Evidence from autoradiographic, in situ hybridization, immunostaining, and electrophysiological analyses indicates that AT1Rs are localized on parvocellular neurons in the PVN. By contrast, there is little evidence that magnocellular neurons contain AT1Rs. In addition, although it is known that Ang II elicits AT1R-mediated excitation of PVN neurons, including those that innervate the RVL, the location of these AT1Rs (presynaptic or postsynaptic, and on which neuronal phenotype) is not established. Electrophysiological data support a presynaptic location of AT1Rs in the PVN, but evidence for a postsynaptic location of these sites, especially on neurons that innervate brain stem or spinal cord cardiovascular control centers, is lacking.

Because previous studies demonstrated that many of the sympathetic regulatory neurons that project from the PVN to the RVL are glutamatergic, we focused on PVN glutamate neurons as a possible postsynaptic location of AT1R. The present data provide evidence that a population of AT1Rs is located postsynaptically on PVN neurons that contain the glutamate synthetic enzyme phosphate-activated glutaminase (PAG) and that certain of these AT1R-bearing neurons innervate the RVL.

Materials and Methods

Animals and Chemicals
Adult male Sprague-Dawley (SD) rats were obtained from Charles River Laboratories (Wilmington, MA). Rats were kept on a 12-hour light/dark cycle in a climate-controlled room. Rat chow and water were provided ad libitum. Animal use protocols were approved by the institutional animal care and use committee of the University of Florida. Details of the chemicals used in this study are available in the online Data Supplement at http://hyper.ahajournals.org.

Hypothalamic or Cerebral Cortex Neuronal Cultures
Neuronal cultures were prepared from a block of hypothalamic tissue containing the PVN or from the parietal cortex of 5- to 7-week-old SD rats, as detailed in the online Data Supplement.

Recombinant AAV2 Vector
Construction of the recombinant vector AAV2-PAG-eGFP-WPRE [Woodchuck hepatitis post-transcriptional regulatory element] was performed as detailed in the online Data Supplement. PAG is a key enzyme in glutamate synthesis, catalyzing the conversion of glutamine to glutamate in neurons. The PAG promoter drives gene expression primarily within glutamate neurons, as explained in the Methods section. The first approach was to microinject AAV2-PAG-eGFP into the PVN, and after 10 to 14 days, to isolate cells from the PVN and use single-cell RT-PCR and electrophysiology to determine whether individual green fluorescent neurons in culture (PAG positive) contain functional AT1R. In a second complementary approach, we again microinjected AAV2-PAG-eGFP into the PVN and then used immunostaining to demonstrate that the green fluorescent neurons in the PVN in situ contain AT1Rs. In addition, in this latter approach, we used retrograde labeling techniques to demonstrate that certain of the GFP-expressing/AT1R-positive neurons in the PVN project to the RVL.

Neuronal Cultures From Adult Rat Hypothalamic Tissue Containing the PVN
It was first essential to establish the procedures for isolation and culture of neurons from adult rat brain tissue containing the PVN and their characterization with respect to expression of AT1Rs and PVN-typical neurotransmitters. A block of hypothalamic tissue containing the PVN was dissected from 4 Sprague-Dawley rats, tissue pooled, and cells isolated and cultured for 4 days, as detailed in the Methods section. Approximately 40% of the isolated/cultured cells exhibited neuron-like morphology (Figure S1A), and their presence was confirmed by immunostaining using antibodies against the neuron-specific marker NeuN (Figure S1B). The remaining cells in these cultures were mostly astroglia, as evidenced by the presence of immunoreactive glial fibrillary acidic protein (Figure S1B). Real-time RT-PCR analysis of the

Electrophysiological Recordings
Recordings of delayed rectifier K+ current (I\(_{\text{Kv}}\)) from PVN neurons in culture were made using whole-cell patch-clamp procedures in the voltage-clamp mode, as detailed in the online Data Supplement.

Real-Time RT-PCR
Levels of mRNA of AT1R, PAG, 67-kDa glutamic acid decarboxylase (GAD67), AVP, OT, ENK, CRH, and β-actin were analyzed via quantitative real-time RT-PCR using either TaqMan or SYBR green kits (Bio-Rad), as detailed in the online Data Supplement.

Single-Cell RT-PCR and Nested PCR
RT-PCR analysis of AT1R and β-actin in single PVN neurons in culture was performed as described in the online Data Supplement.

Immunocytochemistry
PAG, GAD67, AT1R, neuron-specific nuclear protein (NeuN), and glial fibrillary acidic protein immunostaining was performed as described in the online Data Supplement.

Data Analysis
Data are expressed as mean±SEM. Statistical significance was evaluated using the Student t tests. Differences were considered significant at P<0.05, and individual P values are noted in the figure legends.

Results
The overall goal of this study was to determine whether functional AT1Rs are localized postsynaptically to glutamatergic neurons in the PVN. We used 2 experimental approaches, both of which relied on the use of AAV2-PAG-eGFP to target green fluorescent protein (GFP) expression to PAG-containing neurons in the PVN. The PAG promoter drives gene expression primarily within glutamate neurons, as explained in the Methods section. The first approach was to microinject AAV2-PAG-eGFP into the PVN, and after 10 to 14 days, to isolate cells from the PVN and use single-cell RT-PCR and electrophysiology to determine whether individual green fluorescent neurons in culture (PAG positive) contain functional AT1R. In a second complementary approach, we again microinjected AAV2-PAG-eGFP into the PVN and then used immunostaining to demonstrate that the green fluorescent neurons in the PVN in situ contain AT1Rs. In addition, in this latter approach, we used retrograde labeling techniques to demonstrate that certain of the GFP-expressing/AT1R-positive neurons in the PVN project to the RVL.
hypothalamic brain tissue used for isolation and culture of PVN cells revealed the presence of mRNAs for AVP, OT, ENK, CRH, PAG (used as a glutamate neuron marker), and GAD67 (used as a GABA neuron marker; Figure S2A). This indicates that the hypothalamic block contains transmitters that are typical of the PVN (AVP, OT, ENK, CRH, and glutamate) or which surround the PVN (GABA). Parallel analyses of parietal cerebral cortical tissues revealed the expected high levels of ENK, CRH, PAG, and GAD67, but neither OT nor AVP (Figure S2A), validating the RT-PCR procedure. RT-PCR analyses revealed that all of the above neuron types were maintained in the hypothalamic cells in culture (Figure S2B). The PVN contains a high density of AT1Rs,13–18 and AT1R mRNA was present in the hypothalamic tissue used for the isolation and culture of neurons, as well as in the cultures (Figure S2B and S2C). As expected, the hypothalamic cultures contain greater levels of AT1R mRNA compared with parietal cortex neurons in culture (Figure S2C). Single-cell RT-PCR analysis revealed the presence of the AT1R gene in neurons from a representative dish of cells in culture (Figure S2A). This effect of Ang II was time dependent and was reversed by treatment with the AT1R antagonist losartan (1 μmol/L; Figure S3A and S3B). The data in Figure S3C demonstrate that Ang II produced 14.71±3.02% and 3.02±1.21% decreases in I\textsubscript{Kv}, respectively, in the absence or presence of losartan (cell capacitance was 20.11±1.58 pF; n=7 neurons; P<0.001). Collectively, these data indicate that we are able to isolate and culture neurons from a block of adult rat hypothalamus containing the PVN, that these cultures contain populations of neurons that are typical of the PVN, and that some of the isolated neurons express functional AT1R.

**Localization of AT1R on PAG-Positive Neurons in the PVN**

On the basis of the above experiments, the cells that were isolated and cultured from the block of adult rat hypothalamus containing the PVN include glutamate neurons. To target this neuronal population for further experiments on AT1R localization and Ang II actions, we used the vector AAV2-PAG-eGFP to drive GFP expression primarily within glutamate neurons in the PVN. The AAV2-based vector was highly effective in vivo, because bilateral microinjection of AAV2-PAG-eGFP (200 nL; 1.2×10\textsuperscript{12} genome copies per milliliter) into the PVN of rats elicited significant expression of GFP in this nucleus within 7 to 10 days, and expression persisted for several months after the injection (see representative fluorescence micrograph taken at 10 days after injection of AAV2-PAG-eGFP; Figure 1A). This PAG-driven GFP expression was largely specific to PVN glutamate neurons, as indicated by colabeling with immunoreactive NeuN (neuron-specific marker; Figure 1B and Figure 1C). AAV2-PAG-eGFP-driven expression of GFP within GABA neurons (GAD67 immunopositive) was minimal. GFP expression did not colocalize with GAD67 within the PVN, and any colocalization was restricted to the area surrounding the PVN (Figure 2). Many of the GFP-expressing neurons within the PVN of AAV2-PAG-eGFP–injected rats contain immunoreactive AT1Rs (Figure 3). In addition, a number of the GFP-positive cells in the PVN are devoid of immunoreac-
viable (β-actin positive) green fluorescent neurons revealed that 9 cells (≈30%) expressed AT1Rs on the basis of the presence of a 125-bp AT1R fragment on agarose gels (Figure 4B). Ang II (100 nmol/L) produced a significant (≈16%) decrease in $I_{Kv}$ (current density of 37.51±4.63 pA/pF under control conditions versus 30.73±4.62 pA/pF in the presence of Ang II; $P<0.05$; membrane capacitance [Cm] was 18.22±1.93 pF) in 8 of 16 GFP-positive neurons tested (Figure 4C and 4D). As shown in Figure 4D, 50% of the selected green cells were not responsive to Ang II ($I_{Kv}$, current density of 37.93±6.45 pA/pF under control conditions and 36.7±6.39 pA/pF in the presence of Ang II; $P>0.05$; Cm was 19.49±1.89 pF). This inhibitory action of Ang II on $I_{Kv}$ in the GFP-expressing neurons was abolished by 1 μmol/L of losartan (Figure 4E). Collectively, the data from Figures 1 through 4 suggest that a population of GFP-expressing (PAG-positive) neurons within the PVN contain functional AT1Rs. Considering the locus of the microinjections and the GFP expression and the fact that few of the GFP-positive neurons are GABAergic, it is likely that the majority of GFP-expressing (PAG-positive) neurons are glutamatergic.

**Discussion**

The major findings of the present study are that AT1Rs are located on PAG-positive neurons within the PVN and that certain of these AT1R-bearing neurons innervate the RVLM. Although we cannot exclude the possibility that some of the
neurons identified as AT1R containing are GABAergic, it is probable that the majority of these cells are glutamatergic, because the AAV2-PAG-eGFP vector appears to drive GFP expression almost entirely in the latter type of neuron when injected into the PVN.

The significance of these results is straightforward. The PVN has a key role in the CNS control of sympathetic outflow and blood pressure, as well as in regulating the activity of the hypothalamic-pituitary axis. The PVN contains high densities of AT1Rs, mostly located within the

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**Figure 3.** Immunoreactive AT1Rs on PAG-positive neurons in the PVN. A, Representative fluorescence micrograph of GFP expression in the PVN 10 days after microinjection of AAV2-PAG-eGFP. B, Grayscale view of panel A indicating the location of the third cerebroventricle (3V). C, Localization of AT1R-immunoreactive cells within the same field as in panel A. D, G+A indicates overlap of GFP and AT1R. Data are representative of 4 rats. Bar, 100 μm.

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**Figure 4.** Functional AT1R on PAG-specific PVN neurons. A, Representative micrographs of GFP-expressing neurons dissociated from a rat that was injected previously in the PVN with AAV2-PAG-eGFP. Left to right, Phase-contrast image; GFP; immunoreactive PAG (red); and P+G (overlap of GFP and PAG). Bar, 100 μm. B, Presence of AT1R mRNA in a population of PAG-positive neurons. Ethidium bromide–stained gel showing RT-PCR products from 7 single GFP (PAG) -expressing PVN neurons in culture. Top, β-Actin mRNA. Bottom, AT1R mRNA is present in cells 1, 3, 5, and 6. L indicates 100-bp DNA ladder; P, positive control, mRNA purified from PVN; N, negative control, RNase-free water instead of the sample mRNA. C, top, Representative current tracings showing inhibition of $I_{Kv}$ by Ang II (100 nmol/L) in GFP (PAG) -expressing neurons. Control recordings (Con; black) were made before the application of Ang II (red), followed by washout of Ang II (Wash; pink). Bottom, Blockade of the inhibitory effect of Ang II on $I_{Kv}$ by 1 μmol/L of losartan (Los). Los alone, black; Los +Ang II, Red. D, Ang II decreased neuronal $I_{Kv}$ in 8 of 16 neurons (Resp indicates responders). Bar graphs are mean±SEM. * $P<0.05$. E, The inhibitory effects of Ang II were blocked by Los (1 μmol/L). Data are mean±SEM from 5 neurons. * $P<0.05$. 

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and blood pressure. Data also suggest that Ang II can act via CNS-mediated stimulatory effects on sympathetic outflow. The actions of Ang II via these receptors contributes to its pituitary axis. Furthermore, experimental models of hypertension, such as the spontaneously hypertensive rat, exhibit increased levels and activity of AT1Rs in the PVN. Thus, it is essential to understand the specific location of AT1Rs within the PVN, and which neuronal pathways are regulated by Ang II within this nucleus. The current findings, which provide the first definitive evidence that AT1Rs are located on PAG-containing neurons in the PVN, are a significant step forward in this regard. Nonetheless, the results also raise many questions.

One primary issue concerns the discrete location of the AT1Rs on glutamate neurons, whether they occur presynaptically, postsynaptically, or in both locations. Several previous studies have provided good evidence that Ang II acts at presynaptic AT1Rs in the PVN, located either on glutamatergic interneurons or on GABAergic neuron terminals. It has also been demonstrated that Ang II elicits AT1R-mediated excitation of PVN neurons that innervate the RVLM, but these studies did not define the synaptic location of this particular set of AT1Rs. Data from the present study indicate the presence of immunoreactive AT1Rs on at least some of the PAG-positive neurons that project from PVN to RVLM (Figure 5). In addition, we have recorded electric responses after AT1R stimulation in PAG-containing neurons isolated from the PVN, demonstrated AT1R mRNA expression within these neurons, and shown the presence of AT1R immunoreactivity on the cell bodies of GFP-expressing (PAG) neurons in brain sections (Figures 3 and 4). Collectively, these data suggest a postsynaptic location of a population of functional AT1Rs on PAG-containing neurons in the PVN, including those that project to the RVLM. Although our experiments have focused on the PVN neurons that project to the RVLM, it is established that other PVN neuron populations can influence sympathetic outflow by projecting directly to preganglionic sympathetic neurons in the intermediolateral column of the spinal cord and that some PVN-spinal neurons branch to innervate neurons in the RVLM. Thus, it will also be interesting to determine whether these populations of PVN neurons are PAG positive and contain AT1R.

Many neurons within the CNS (including the PVN) contain cotransmitters. Thus, although our data indicate the presence of AT1Rs on PAG-positive (mostly glutamate) neurons in the PVN, it is very likely that these neurons also contain ≥1 other transmitter. One possible candidate is CRH, because previous studies have demonstrated that PVN neurons that express CRH and project to the median eminence also express AT1R mRNA and immunoreactive AT1Rs. With respect to the AT1R-containing PAG-positive neurons that project from the PVN to the RVLM, we do not know whether they contain a cotransmitter or, if so, the identity. However, a preliminary single-cell PCR screen of the GFP-expressing (PAG-positive) PVN neurons within the cultures has revealed that many are oxytocinergic. One future direction will be to solidify these data and further assess the phenotypic nature of the AT1R-containing PVN neurons. Such an effort will be made easier if specific promoters that can be used to drive gene expression within OT or CRH neurons become available.

Our ability to drive gene expression within PAG-positive neurons in the PVN and the present demonstration that certain of these neurons contain AT1Rs open up new avenues of investigation concerning the regulation of Ang II actions at this site. Namely, in previous studies, we have demonstrated that macrophage migration inhibitory factor serves as a negative regulator of the AT1R-mediated actions of Ang II at the PVN. The current results and the development of a vector for gene transduction within PAG-positive neurons give us the rationale and means for determining the effects of overexpression of macrophage migration inhibitory factor specifically in these neurons in the PVN on Ang II–induced cardiovascular responses.

In summary, the results presented here provide the first evidence that AT1Rs are located on PAG-positive (likely glutamatergic) neurons in the PVN and that certain of these AT1R-bearing neurons innervate the RVLM. Furthermore,
our data provide evidence that a population of AT1Rs is located postsynaptically. As such, the data are a first step to understanding which specific neuronal pathways in the PVN are modulated by Ang II, and they open the door to studies that target each specific pathway and how it contributes to the CNS-mediated cardiovascular actions of this peptide.

Perspectives
Knowledge of which neuronal pathways in the PVN are influenced by Ang II/AT1R interactions, and how these Ang II actions are regulated, is essential to understanding the CNS mechanisms that are involved in blood pressure regulation and neurogenic hypertension. Although it is known that the PVN contains AT1Rs and that these receptors have a role in blood pressure control and hypertension, evidence concerning their discrete localization within this hypothalamic nucleus is lacking. The present study gives a clear demonstration of the presence of functional AT1Rs on PAG-positive neurons in the PVN, including those that project to the RVLM. As such, this study provides information that is fundamental to our understanding of the CNS control of blood pressure.

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Disclosures
None.

References