Angiotensin depolarizes parvocellular neurons in paraventricular nucleus through modulation of putative nonselective cationic and potassium conductances

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Latchford, Kevin J., and Alastair V. Ferguson. Angiotensin depolarizes parvocellular neurons in paraventricular nucleus through modulation of putative nonselective cationic and potassium conductances. Am J Physiol Regul Integr Comp Physiol 289: R52–R58, 2005. First published April 14, 2005; doi:10.1152/ajpregu.00549.2004.—Neurosecretory parvocellular neurons in the hypothalamic paraventricular nucleus (PVN) exercise considerable influence over the adenohypophysis and thus play a critical role in neuroendocrine regulation. ANG II has been demonstrated to act as a neurotransmitter in PVN, exerting significant impact on neuronal excitability and also influencing corticotropin-releasing hormone secretion from the median eminence and, therefore, release of ACTH from the pituitary. We have used whole cell patch-clamp techniques in hypothalamic slices to examine the effects of ANG II on the excitability of neurosecretory parvocellular neurons. ANG II application resulted in a dose-dependent depolarization of neurosecretory neurons, a response that was maintained in tetrodotoxin (TTX), suggesting a direct mechanism of action. The depolarizing actions of this peptide were abolished by losartan, demonstrating these effects are AT1 receptor mediated. Voltage-clamp analysis using slow voltage ramps revealed that ANG II activates a voltage-independent conductance with a reversal potential of $-37.8 \pm 3.8\, \text{mV}$, suggesting ANG II effects on a nonselective cationic current. Further, a sustained potassium current characteristic of $I_K$ was significantly reduced (29.1 \pm 4.7\% by ANG II. These studies identify multiple postsynaptic modulatory sites through which ANG II can influence the excitability of neurosecretory parvocellular PVN neurons and, as a consequence of such actions, control hormonal secretion from the anterior pituitary.

neuroendocrine regulation; electrophysiology; ion channels

THE PARAVENTRICULAR NUCLEUS of the hypothalamus (PVN) plays essential roles in neuroendocrine and autonomic regulation (45). Extensive anatomic analysis has resulted in a thorough classification of this nucleus’s architecture resulting in the traditional view of PVN as a nucleus consisting predominantly of magnocellular and parvocellular neurons. Magnocellular neuroendocrine cells are recognized for their role in the production, storage, and secretion of the peptide hormones vasopressin and oxytocin (45). Parvocellular neurons, on the other hand, consist of two discrete subdivisions: preautonomic cells (nonneurosecretory), which play integral roles in the control of autonomic output through descending connections to the medulla and spinal cord and neuroendocrine (neurosecretory) cells, which are involved in the regulation of the anterior pituitary via projections to the median eminence (45).

For more than a decade the electrophysiological properties of these neurons have been used as an experimental tool to permit differential analysis of these subpopulations of neurons (18, 46). Although the description of magnocellular neurons has remained relatively unchanged, considerable debate has emerged concerning the electrical profiles of the neurosecretory and nonneurosecretory parvocellular subdivisions. Luther et al. (31) demonstrate that the electrical criterion traditionally cited as being characteristic of parvocellular neurons is consistent with preautonomic parvocellular neurons projecting to the spinal cord. Specifically, nonneurosecretory neurons demonstrate a low-threshold depolarization (typically generating one or two action potentials) after a hyperpolarizing current pulse, consistent with the generation of a prominent low-threshold spike (LTS) and a robust T-type calcium current. In contrast, neurons that project to the median eminence have a separate electrophysiological identity that is characterized by a lack of prominent LTSs and a relatively small T-type calcium current. In accordance with this view, Stern observed that neurons in the PVN retrogradely labeled from preautonomic nuclei were found to express LTSs and an inwardly rectifying $I-V$ relationship, as described above (41). Additionally, Cui et al. (6) demonstrated that paraventricular neurons containing a retrograde label transported from the thoracic [T(1)-T(4)] intermediolateral column displayed a hyperpolarization-activated inward rectification and a LTS consistent with the preautonomic neurons characterized by Stern (41). As a consequence of these observations, the traditional models of PVN input/output and the regulation of its primary cell types by both classical neurotransmitters and neuropeptides have been modified to reflect the increasingly recognized heterogeneity of this nucleus.

ANG II, a hormone traditionally recognized for its peripheral endocrine roles in the regulation of vascular resistance and control of fluid-electrolyte homeostasis has been suggested to act as a neurotransmitter regulating the excitability of PVN neurons. Angiotensinergic fibers, cell bodies, and receptors have been reported in PVN (22, 23, 25, 30, 36, 39), and ANG II has been shown to influence a variety of neuroendocrine and autonomic functions (7, 11, 12, 23). Early reports have focused primarily on ANG II actions on magnocellular neurons (9, 28, 29, 38). Interestingly, Lenkei et al. (23–25) observed a high level of AT1 receptor mRNA expression within the parvocellular areas of PVN and the surrounding periventricular area, but no expression in the magnocellular PVN.

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Intriguingly, Oldfield et al. (34) demonstrate that the distribution of AT₁ receptor correlates strongly with neurons in the anterior parvocellular division of PVN, which direct axons to the median eminence (34). In addition, intracerebroventricular injection of ANG II stimulates ACTH release (15, 40), not a surprising discovery in view of the observation that AT₁ receptor mRNA is localized in corticotropin-releasing hormone (CRH) containing neurons (1). Indeed, activation of known ANG II-positive subfornical organ (SFO) efferents to PVN results in an elevation of hypophysical-portal plasma CRH levels and increased circulating ACTH (37). The ionic mechanisms by which ANG II mediates its effects upon parvocellular neurosecretory neurons, however, remain unresolved.

We have used the whole cell patch-clamp technique to characterize the actions of ANG II on neurosecretory parvocellular neurons. We report that application of ANG II results in an excitation of these neurons, which is mediated directly by AT₁ receptors. Moreover, the excitation is likely the result of the activation of a nonselective cationic conductance and/or the inhibition of the delayed rectifier potassium current.

**MATERIALS AND METHODS**

**Slice preparation.** Experiments were performed using hypothalamic slices prepared, as previously described (29). Briefly, male Sprague-Dawley rats (150–250 g, Charles River, Quebec, Canada) were killed by decapitation, and the brain was quickly removed from the skull and immersed in cold (1–4°C) artificial cerebrospinal fluid (aCSF). The brain was blocked and 400-μm coronal sections were formed using the whole cell configuration of the patch-clamp technique. Losartan (generously provided from Dupont Pharmaceuticals, Wilmington, DE) was made daily to the required concentration.

**Electrophysiology.** Electrophysiological experiments were performed using the whole cell configuration of the patch-clamp technique to record from PVN neurons. Patch pipettes were pulled to a resistance of 5–7 MΩ and filled with the pipette solution described below. Seal resistance was at least 1 GΩ and as large as 10 GΩ. Signals were processed with an Axoclamp-2A amplifier. An Ag-AgCl electrode connected to the bath solution via a KCl-agar bridge served as reference. All signals were digitized using the Cambridge Electronic Design 1401 plus interface and stored on computer for off-line analysis. Drugs were applied by switching the perfusion solution from aCSF to a solution containing the desired drug. Mean group values were compared with an unpaired t-test for those neurons where t-test was observed, indicating that these neurons do not show significant desensitization to ANG II.

**Solutions.** The aCSF composition was (in mM): 124 NaCl, 2 KCl, 1.25 KPO₄, 2 CaCl₂, 1.3 MgSO₄, 20 NaHCO₃, and 10 glucose. Osmolarity was maintained between 285 and 300 mosM and pH between 7.3 and 7.4. The pipette solution contained (in mM): 140 potassium glutonate, 0.1 CaCl₂, 1.1 EGTA, 10 HEPES, and 2 Na ATP, and had a pH of 7.25 (adjusted with KOH if necessary).

**RESULTS**

Whole cell patch-clamp recordings were obtained from a total of 80 putative neurosecretory parvocellular PVN neurons. These neurons had a mean resting membrane potential (RMP) of −58.5 ± 1.9 mV (means ± SE), displayed action potentials with a minimum-spike amplitude of 60 mV, and had a mean input resistance of 1,155 ± 75 MΩ. Neurosecretory parvocellular neurons expressed neither a prominent I_A nor a LTS as reported by Luther et al. (31). Neurons that displayed a prominent “A current” following hyperpolarizing current pulses and had a linear I-V relationship were classified as magnocellular neurons, while those neurons that featured a low-threshold calcium conductance and whose I-V demonstrated an inward rectification at hyperpolarized potentials were classified as nonneurosecretory preautonomic parvocellular (41, 46). Neither group of neurons was considered further in the present study.

**ANG II depolarizes neurosecretory PVN neurons.** A total of 57 (RMP: −57.0 ± 1.5 mV) neurosecretory parvocellular neurons were tested for the effects of bath application of ANG II using current-clamp techniques. After a control recording period of at least 5 min, ANG II was administered by bath perfusion in concentrations ranging from 0.01 μM to 10 μM for a period of 30 s. Of the 57 neurosecretory neurons tested with ANG II, 38 (67%) depolarized, 3 (5%) hyperpolarized, and 16 (28%) were unaffected by peptide administration, as illustrated in Fig. 1. These depolarizing responses were dose dependent [10.0 μM, 10.0 ± 1.5 mV (n = 6); 1.0 μM, 8.8 ± 0.5 mV (n = 7); 0.1 μM, 6.7 ± 0.5 mV (n = 21); 0.01 μM, 0.8 ± 0.8 mV (n = 4)] and followed a sigmoidal dose-response relationship with an estimated EC₅₀ of 5.5 × 10⁻⁸. In some cases, after recovery of the neuron to resting RMP, ANG II application was repeated and a second depolarization [ANG II 0.1 μM, 6.9 ± 1.0 mV vs. 6.2 ± 0.9 mV (n = 4); P > 0.5, paired t-test] was observed, indicating that these neurons do not show significant desensitization to ANG II.

The effect of ANG II on the input resistance of neurosecretory neurons was also determined in 10 cells, which responded to ANG II with a depolarization. In the majority of neurons (n = 7), ANG II (0.1 μM) decreased input resistance (−283 ± 34 MΩ: Control, 1,030 ± 120 MΩ; ANG II, 747 ± 88 MΩ), while the remaining three cells were unaffected. The reversal potential (E₉ₑᵥ) of the underlying conductance responsible for the actions of ANG II on these neurons was extrapolated from an I-V relationship. In those neurons that demonstrated a depolarizing response concomitant with a decrease in input resistance following ANG II application, the E₉ₑᵥ was found to be −45.5 ± 3.5 mV (n = 7), suggesting potential effects on a nonselective cationic conductance (Iᵠₛcdc).

**ANG II’s effects on neurosecretory neurons are maintained in TTX.** Several recent reports have documented that the effects of a number of well-known neuropeptides on PVN neurons, including ANG II, adrenomedullin, AVP, orexin, as well as norepinephrine, are dependent on a modulation of synaptic input to these neurons. The effects of ANG II before and after bath application of TTX on neurosecretory neurons were therefore evaluated to determine whether the ANG II response in these neurons was maintained in synaptic isolation. The results of these experiments are shown in Fig. 2, A and B. The excitatory effects of ANG II on neurosecretory neurons were
maintained in TTX [0.1 μM ANG II: 6.6 ± 0.6 mV vs. 1.0 μM TTX/ANG II: 6.2 ± 0.3 mV (n = 6); P > 0.5, paired t-test], suggesting a direct interaction of ANG II on the membranes of these cells that is independent of a change in synaptic input.

**ANG II effects on neurosecretory neurons are losartan sensitive.** Neuroanatomic studies indicate a high degree of AT1 receptor mRNA localization in the parvocellular region of the PVN (23–25), particularly neurosecretory CRH neurons. The actions of ANG II were therefore studied in the presence of the nonpeptidergic AT1 receptor-specific antagonist losartan to determine whether this receptor was indeed responsible for the observed depolarizations (Fig. 2, C and D). The effects of ANG II on this subset of parvocellular neurons are abolished following bath application of 1.0 μM losartan [0.1 μM ANG II: 7.2 ± 0.4 mV vs. losartan/ANG II: 1.4 ± 0.7 mV (n = 5); P < 0.001, paired t-test], demonstrating the AT1 receptor-mediated nature of these depolarizations.

**ANG II activates a putative nonselective cationic current in neurosecretory neurons.** Voltage-clamp experiments were conducted to determine the identity of the current responsible for the effects of ANG II on neurosecretory PVN neurons. Our input resistance data suggested the likely conductance was a nonselective cationic current (INSCC) based on the observed $E_{rev}$ extrapolated from $I-V$ relationships taken during the depolarizations. This hypothesis is in accordance with previous studies documenting that the depolarizing effects of ANG II and other peptides on PVN neurons are mediated by the activation of INSCC (13, 14, 47). We thus examined the effects of ANG II on this current in voltage clamp after application of TTX (1.0 μM) to block voltage-gated sodium channels. Bath application of 0.1 μM ANG II during slow (10 mV/s) depolarizing voltage ramps (−80 to +20 mV) revealed an increase in conductance over this voltage range in 7 of 12 (58%) neurosecretory neurons tested (Fig. 3). The proportion of ANG II effects on voltage ramps was found to be similar to the observed depolarizing effects (Chi-square, $P > 0.5$) of ANG II in current clamp. The subtracted current was linear over the voltage range −80 to +20 mV (Fig. 3B, inset), suggesting activation of a voltage-independent current with a mean reversal potential of −37.8 ± 3.8 mV (Fig. 3B), consistent with activation of INSCC.

**ANG II inhibits $I_K$ in neurosecretory neurons.** Interestingly, several of the ramps revealed that the difference in current between control and ANG II application was nonlinear in the 0 to +20 mV voltage range, suggesting the influence of an inwardly rectifying current. Our input resistance data estimated
a reversal potential of $-45.5 \pm 3.5$ mV, which is lower than the reversal potential elicited by the $I_{NSCC} (-37.8 \pm 3.8$ mV). These observations in combination with the demonstration that ANG II has effects on the delayed rectified potassium current ($I_{K}$) (3, 17, 49) lead us to suspect this current was modulated by ANG II. This hypothesis was tested using a voltage step protocol (250-ms steps from $-60$ to $+20$ mV were used) in the presence of TTX. Bath application of 0.1 μM ANG II caused a significant decrease in $I_{K}$ ($29.1 \pm 4.7\%$, Fig. 4) in four of seven ($P < 0.005$, paired t-test) neurosecretory neurons as tested at the $+20$-mV step. The proportion of ANG II effects on $I_{K}$ was found to be similar to the observed depolarizing effects (Chi-square, $P > 0.5$) of ANG II in current clamp. Although no recovery was seen from this decrease in current, this effect was unlikely to be the result of current run-down as application of 1 nM ANG II (a dose that does not result in a depolarization) had no significant effect on $I_{K}$ ($n = 4$, data not shown).

**DISCUSSION**

The complexity of PVN when combined with the difficulty in selectively studying one population of cells has made it difficult to thoroughly characterize each of its cellular subtypes. Therefore, although the electrophysiological properties of PVN neurons have been studied in detail, only two broad categories are traditionally cited: magnocellular (Type I) and parvocellular (Type II) neurons. Whole cell patch-clamp techniques in combination with sophisticated immunohistochemical identification of neurons have permitted a rigorous characterization of PVN neurons.

Recently, Stern (41) characterized the cellular properties of preautonomic nonneurosecretory neurons in PVN by combining in vivo retrograde tracing techniques with in vitro patch-clamp recordings. Subsequently, Luther et al. (31) documented using intravenous injections of the retrograde tracer flouro-gold that neurosecretory neurons whose projections extended to the median eminence had unique electrophysiological fingerprints of their own (31). These two studies, in combination, demonstrated that neurosecretory neurons were without a prominent LTS and a robust T-type calcium current, whereas nonneurosecretory neurons were indistinguishable from traditional Type II parvocellular neurons in that they generated a prominent LTS and a robust T-type calcium current. We have used these obser-

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**Fig. 2.** ANG II-mediated depolarization of neurosecretory parvocellular neurons is maintained in tetrodotoxin (TTX) and abolished by losartan. (A) Current clamp recording illustrates that ANG II (0.1 μM, 30 s, application indicated by bar)-induced depolarization of a neurosecretory parvocellular neuron is maintained after pretreatment with 1.0 μM TTX (application indicated by light-gray extended bar). Action potentials have been truncated. (B) Bar graph summarizes the effects of ANG II on neurosecretory parvocellular neurons after application of TTX. (C) Current clamp recording illustrating that the 0.1 μM ANG II (30 s, application indicated by bar)-induced depolarization of a neurosecretory parvocellular neuron is significantly reduced by pretreatment with 1.0 μM losartan (application indicated by white extended bar). Recordings are made from the same neuron ~20 min apart. (D) Bar graph summarizes the effects of ANG II on neurosecretory parvocellular neurons after application of losartan.
We report here that ANG II excites neurosecretory neurons in a dose-dependent fashion, which does not rely on synaptic input, as the depolarizations are maintained in synaptic isolation. In addition, the excitation is abolished by the AT1 antagonist losartan, demonstrating that ANG II’s effects on this subset of PVN neurons are mediated by the AT1 receptor. These observations are particularly intriguing given that the majority of studies thus far have concentrated on the actions of ANG II on magnocellular neurons (9, 28, 29, 38). These reports have themselves garnered considerable debate with respect to AT1 receptor localization with PVN. The autoradiographic distribution of labeled ANG II as an indication of ANG II binding sites within the central nervous system (16, 19, 32) includes the SFO, median eminence, supraoptic nucleus (SON), and PVN complementary sites to this peptide’s actions in cardiovascular regulation. Interestingly, high levels of AT1 receptor mRNA expression within the parvocellular areas of PVN and the surrounding periventricular area have been documented, but the magnocellular region of PVN is devoid of such mRNA, providing an interesting caveat to those observations (23–25), detailing the role of ANG II in PVN. Indeed, recent immunohistochemical studies have observed that AT1 receptors are found within specific regions of parvocellular PVN where neurosecretory neurons are localized (34).

The observation that the ANG II-mediated depolarization of neurosecretory neurons was maintained in TTX indicates that ANG II exerts direct effects on these cells. Similar direct actions of ANG II on other neuronal populations have also been described, and two specific conductances, INSCC and IK, have frequently been implicated as underlying these excitatory effects (2, 10, 42, 43, 48).

In our experiments the ANG II-induced current (measured using voltage ramps), was voltage independent between −80 and −20 mV and showed a reversal potential similar to previously characterized NSCCs (2). These observations support the conclusion that ANG II influences NSCC in these neurons. In accordance with these findings, recent studies have also demonstrated ANG II’s effects on PVN neurons that project to the rostral ventrolateral medulla, which also appear...
to be the result of modulation of a cationic conductance (5). However, the more depolarized reversal potential (+2 mV) for ANG II’s effects reported in these studies suggests they are not mediated by the same channel in these anatomically adjacent neurons. ANG II has, however, been reported to modulate NSCCs with reversal potentials similar to those we observe in SON (48), SFO (35), and diagonal band of Broca (2) neurons.

The effects of ANG II on the voltage-dependent delayed rectifier (I\textsubscript{K}), a current critical in neuronal repolarization have also been examined in a number of studies, although different effects have been reported in different cell types. Our observation that inhibitory effects of ANG II on this channel are in accordance with those of Sumners et al. who demonstrated similar AT\textsubscript{1} receptor-mediated actions of ANG II in newborn hypothalamic/brain stem cultures (44). In their studies, the reduction was shown to be dependent on a Ca\textsuperscript{2+}/calmodulin/CaM K\textsubscript{II} signaling pathway, as well as protein kinase C (42). Nagamoto et al. (33) have also found that in a small population of SON neurons, I\textsubscript{K} was weakly sensitive to ANG II, while our own previous recordings (29) from magnocellular PVN neurons in hypothalamic slices reported no effects of ANG II on this conductance.

In putative preautonomic PVN neurons, ANG II has been demonstrated to cause a shift in the activation curve of a hyperpolarization-activated current (I\textsubscript{h}), which was mediated by the AT\textsubscript{1} receptor (8). We did not, however, observe a substantial I\textsubscript{h} in neurosecretory parvocellular neurons, and therefore, effects on this conductance probably do not contribute significantly to ANG II actions on these cells. A number of studies have examined indirect mechanisms that contribute to the effects of ANG II on the PVN, as well as other groups of CNS neurons. In magnocellular neurons we have shown that ANG II-induced depolarizations are dependent on activation of glutamate interneurons (21) and also that ANG II increases inhibitory post synaptic current frequency through activation of a nitric oxide-driven feedback loop (20), suggesting that complex intranuclear interactions underlie the final integrated actions of ANG II in PVN. In contrast, Li and colleagues (26, 27) have demonstrated that ANG II stimulates parvocellular PVN neurons projecting to either the spinal cord or the rostral ventrolateral medulla by presynaptic AT\textsubscript{1} receptor-mediated attenuation of GABAergic synaptic inputs. Presynaptic actions of ANG II have also been demonstrated in the nucleus tractus solitarius where Barnes et al. (4) have demonstrated that excitatory effects of ANG II are at least, in part, the result of presynaptic effects on glutamatergic neurotransmission. In combination, this literature suggests that both pre- and postsynaptic actions of ANG II may contribute to the coordinated postsynaptic actions of this peptide in controlling the excitability of different populations of PVN output neurons.

In summary, we have shown that ANG II excites neurosecretory parvocellular neurons likely through a combination of activation of a putative NSCC and inhibition of I\textsubscript{K}. These findings are consistent with previous studies investigating the postsynaptic actions of ANG II on hypothalamic neurons. Such effects of ANG II in modulating specific ion channels, which control the excitability of neuroendocrine PVN cells, highlight cellular mechanisms through which ANG II may influence the secretion of anterior pituitary hormones.

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**References**

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