Angiotensin Depolarizes Parvocellular Neurons in Paraventricular Nucleus through Modulation of Putative Non-Selective Cationic and Potassium Conductances

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Running Title: Angiotensinergic effects on neurosecretory PVN neurons

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Abstract

Neurosecretory parvocellular neurons in the hypothalamic paraventricular nucleus (PVN) exercise considerable influence over the adenohypophysis and thus play a critical role in neuroendocrine regulation. Angiotensin II (AII) has been demonstrated to act as a neurotransmitter in PVN exerting significant impact on neuronal excitability and also influencing CRH 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 AII on the excitability of neurosecretory parvocellular neurons. AII application resulted in a dose-dependent depolarization of neurosecretory neurons, a response which was maintained in tetrodotoxin (TTX) suggesting a direct mechanism of action. The depolarizing actions of this peptide were abolished by losartan demonstrating these effects to be AT₁ receptor mediated. Voltage-clamp analysis using slow voltage ramps revealed that AII activates a voltage independent conductance with a reversal potential of -37.8±3.8mV suggesting effects on a non-selective cationic current. Further, a sustained potassium current characteristic of Iₖ was significantly reduced (29.1±4.7%) by AII. These studies identify multiple post-synaptic modulatory sites through which AII can influence the excitability of neurosecretory parvocellular PVN neurons and, as a consequence of such actions, control hormonal secretion from the anterior pituitary.

Keywords: neuroendocrine regulation, electrophysiology, ion channels
Introduction

The paraventricular nucleus of the hypothalamus (PVN) plays essential roles in neuroendocrine and autonomic regulation (45). Extensive anatomical analysis has resulted in a thorough classification of this nucleus’ 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, pre-autonomic cells (non-neurosecretory) 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 over a decade the electrophysiological properties of these neurons have been utilized as an experimental tool to permit differential analysis of these subpopulations of neurons (18; 46). While the description of magnocellular neurons has remained relatively unchanged, considerable debate has emerged concerning the electrical profiles of the neurosecretory and non-neurosecretory parvocellular subdivisions. Luther et al. demonstrate that the electrical criterion traditionally cited as being characteristic of parvocellular neurons is consistent with pre-autonomic parvocellular neurons projecting to the spinal cord (31). Specifically, non-neurosecretory neurons demonstrate a low threshold depolarization (typically generating one or two action potentials) following a hyperpolarizing current pulse consistent with the generation of a prominent low threshold spike and a robust T-type calcium current. In contrast, neurons which project to the median eminence have a separate electrophysiological identity characterized by lack of prominent LTS, and a relatively small
T-type calcium current. In accordance with this view, Stern observed that neurons in PVN retrogradely labeled from pre-autonomic nuclei were found to express low threshold spikes and an inwardly rectifying I-V relationship as described above (41). Additionally, Cui et al. demonstrate 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 (6) consistent with the pre-autonomic neurons characterized by Stern. 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 in order to reflect the increasingly recognized heterogeneity of this nucleus.

Angiotensin II (AII), 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 AII has been shown to influence a variety of neuroendocrine and autonomic functions (7; 11; 12; 23). Early reports have focused primarily on AII actions on magnocellular neurons (9; 28; 29; 38). Interestingly, Lenkei et al. 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 (23-25).

Intriguingly, Oldfield et al. 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, ICV injection of AII stimulates ACTH release (15; 40) not a surprising discovery in view of the observation that AT₁ receptor mRNA is localized in CRH containing neurons (1). Indeed, activation of known AII
positive SFO efferents to PVN results in an elevation of hypophysial-portal plasma irCRF levels and increased circulating ACTH (37). The ionic mechanisms by which AII mediates its effects upon parvocellular neurosecretory neurons however remain unresolved.

We have utilized the whole cell patch clamp technique to characterize the actions of AII on neurosecretory parvocellular neurons. We report that application of AII results in an excitation of these neurons which is mediated directly by AT_1 receptors. Moreover, the excitation is likely the result of the activation of a non-selective 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 (150-250g, Charles River, P.Q., 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 400µM coronal sections were cut through the hypothalamus using a Vibratome. Sections were hemisected, trimmed into blocks containing PVN, and were incubated in oxygenated aCSF (95%O₂/5%CO₂) for at least 90 min. at room temperature. Prior to recording, the slice was transferred into an interface-type recording chamber and continuously perfused with aCSF at a rate of 1ml/min at room temperature.

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 1GΩ and as large as 10GΩ. 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 C.E.D. 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 a Student’s paired “t” test for those neurons where comparisons of two values were obtained in the same cell. Mean group values were compared with an unpaired “t” test when comparing values obtained from two separate populations of neurons.

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 Kgluconate, 0.1 CaCl₂, 1.1 EGTA, 10 HEPES, and 2 NaATP, and had a pH of 7.25 (adjusted with KOH if necessary).

A stock solution of AII (Phoenix Pharmaceuticals, California) was prepared from which daily aliquots were made to the required dilution. Tetrodotoxin (TTX, Alamone Laboratories, Israel) was prepared daily from stock solutions for those experiments where it was necessary to block voltage-activated Na⁺ channels. Losartan (generously provided from Dupont Pharmaceuticals, Delaware) was made daily to the required concentration.
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±1.9mV (mean ± SEM), displayed action potentials with a minimum spike amplitude of 60mV, and had a mean input resistance of 1155±75MΩ. Neurosecretory parvocellular neurons expressed neither a prominent I_A nor a low threshold spike as reported by Luther et al. (31). Neurons which displayed a prominent ‘A current’ following hyperpolarizing current pulses and had a linear I/V relationship were classified as magnocellular neurons while those neurons which featured a low threshold calcium conductance and whose I/V demonstrated an inward rectification at hyperpolarized potentials were classified as non-neurosecretory pre-autonomic parvocellular (41; 46). Neither of these groups of neurons were considered further in the present study.

Angiotensin II Depolarizes Neurosecretory PVN Neurons

A total of 57 (RMP; -57.0±1.5mV) neurosecretory parvocellular neurons were tested for the effects of bath application of AII using current clamp techniques. Following a control-recording period of at least 5 minutes, AII was administered by bath perfusion in concentrations ranging from 0.01µM to 10µM for a period of 30 seconds. Of the 57 neurosecretory neurons tested with AII, 38 (67%) depolarized, 3 (5%) hyperpolarized, and 16 (28%) were unaffected by peptide administration as illustrated in Figure 1. These depolarizing responses were dose dependent (10.0µM; 10.0±1.5mV n=6, 1.0µM; 8.8±0.5mV n=7, 0.1µM; 6.7±0.5mV n=21, 0.01µM; 0.8±0.8mV n=4) and followed a sigmoidal dose response relationship with an estimated EC_{50} of 5.5x10^{-8}. In some cases following recovery of the neuron to resting RMP, AII application was repeated and a second depolarization
Angiotensin II 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 AII, adrenomedullin, AVP, and orexin-A as well as noradrenaline (NA) are dependent upon a modulation of synaptic input to these neurons. The effects of AII before and after bath application of tetrodotoxin (TTX) on neurosecretory neurons were therefore evaluated to determine whether the AII response in these neurons was maintained in synaptic isolation. The results of these experiments are shown in Figure 2A,B. The excitatory effects of AII on neurosecretory neurons were maintained in TTX (0.1µM AII: 6.6±0.6mV vs. 1.0µM TTX /AII 6.2±0.3mV, n=6, p>0.5, paired t-test) suggesting a direct interaction of AII on the membranes of these cells that is independent of a change in synaptic input.

Angiotensin II Effects on Neurosecretory Neurons are Losartan Sensitive
Neuroanatomical 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 AII were therefore studied in the presence of the non-peptidergic AT₁ receptor specific antagonist losartan to determine if this receptor was indeed responsible for the observed depolarizations (Figure 2C,D). The effects of AII on this subset of parvocellular neurons are abolished following bath application of 1.0µM losartan (0.1µM AII: 7.2±0.4mV vs. Losartan/AII 1.4±0.7mV, n=5, p<0.001, paired t-test) demonstrating the AT₁ receptor mediated nature of these depolarizations.

**Angiotensin II Activates a Putative NSCC in Neurosecretory Neurons**

Voltage clamp experiments were conducted in order to determine the identity of the current responsible for the effects of AII on neurosecretory PVN neurons. Our input resistance data suggested the likely conductance was a non-selective cationic current (I_{NSCC}) 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 AII and other peptides on PVN neurons are mediated by the activation of I_{NSCC} (13; 14; 47). We thus examined the effects of AII on this current in voltage clamp following application of TTX (1.0µM) to block voltage gated sodium channels. Bath application of 0.1µM AII during slow (10mV s⁻¹) depolarizing voltage ramps (-80 to +20mV) revealed an increase in conductance over this voltage range in 7 of 12 (58%) neurosecretory neurons tested (Figure 3). The proportion of AII effects on voltage ramps was found to be similar to the observed depolarizing effects (Chi-square, P>0.5) of AII in current clamp. The subtracted current was linear over the voltage range -80 to -20mV (Figure 3A inset) suggesting activation of a voltage independent current with a mean reversal potential of -37.8±3.8mV (Figure 3B), consistent with activation of I_{NSCC}.
Angiotensin II Inhibits I_k in Neurosecretory Neurons

Interestingly, several of the ramps revealed that the difference current between control and AII application was non-linear 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±3.5mV which is lower than the reversal potential elicited by the I_{NSCC} (-37.8±3.8mV). These observations in combination with the demonstration that AII has effects on the delayed rectified potassium current (I_k) (3; 17; 49) lead us to suspect this current was modulated by AII. This hypothesis was tested using a voltage step protocol (250 ms steps from -60 to +20mV were employed) in the presence of TTX. Bath application of 0.1µM AII caused a significant decrease in I_k (29.1±4.7%, Figure 4) in 4 of 7 (P<0.005, paired t-test) neurosecretory neurons as tested at the +20 mV step. The proportion of AII effects on I_k was found to be similar to the observed depolarizing effects (Chi-square, P>0.5) of AII 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 1nM AII (a dose which 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 while 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 characterized the cellular properties of pre-autonomic non-neurosecretory neurons in PVN by combining in vivo retrograde tracing techniques with in vitro patch clamp recordings (41). Subsequently, Luther et al. documented utilizing IV 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 studies in combination demonstrated that neurosecretory neurons were without a prominent LTS and a small T-type calcium current while non-neurosecretory neurons were undistinguishable from traditional Type II parvocellular neuron in that they generated a prominent low threshold spike and a robust T-type calcium current. We have used these observations to characterize the effects of AII on neurosecretory parvocellular neurons.

We report here that AII excites neurosecretory neurons in a dose-dependent fashion that 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 the AII 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 AII 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 AII as an indication of AII binding sites within the CNS (16; 19; 32) includes the SFO, ME, SON, and PVN complementary sites to this peptides 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 AII 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 AII mediated depolarization of neurosecretory neurons was maintained in TTX indicates that AII exerts direct effects on these cells. Similar direct actions of AII on other neuronal populations have also been described, and two specific conductances, INSCC and I_K, have frequently been implicated as underlying these excitatory effects (2; 10; 42; 43; 48).

In our experiments the AII 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 AII influences NSCC in these neurons. In accordance with these findings recent studies have also demonstrated AII effects on PVN neurons which project to the rostral ventrolateral medulla (RVLM), which also appear to be the result of modulation of a cationic conductance (5). However, the more depolarized reversal potential (+2mV) for AII effects reported in these studies suggests they are not mediated by the same channel in these anatomically
adjacent neurons. AII has however been reported to modulate NSCCs with reversal potentials similar to those we observe in supraoptic (48), subfornical organ (35) and diagonal band of broca (2) neurons.

The effects of AII on the voltage dependent delayed rectifier (I_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 of inhibitory effects of AII on this channel are in accordance with those of Sumners et al. who demonstrated similar AT1 receptor mediated actions of AII in newborn hypothalamic/brain stem cultures (44). In their studies the reduction was shown to be dependent on a Ca^{2+}/calmodulin/CaM KII signaling pathway as well as PKC (42). Nagatomo et al. have also found that in a small population of SON neurons I_K was weakly sensitive to AII (33), while our own previous recordings from magnocellular PVN neurons in hypothalamic slices reported no effects of AII on this conductance (29).

In putative pre-autonomic PVN neurons AII has been demonstrated to cause a shift in the activation curve of a hyperpolarization-activated current (I_h), which was mediated by the AT1 receptor (8). We did not however observe a substantial I_h in neurosecretory parvocellular neurons, and therefore effects on this conductance probably do not contribute significantly to AII actions on these cells.

A number of studies have examined indirect mechanisms which contribute to the effects of AII on PVN as well as other groups of CNS neurons. In magnocellular neurons we have shown that AII induced depolarizations are dependent on activation of glutamate interneurons (21), and also that AII increases IPSC frequency through activation of a nitric oxide driven feedback loop (20), suggesting that complex intranuclear interactions underlie the final integrated actions of AII in PVN. In contrast Li et al. have demonstrated that AII
stimulates parvocellular PVN neurons projecting to either the spinal cord or the rostral ventrolateral medulla by presynaptic \( AT_1 \) receptor mediated attenuation of GABAergic synaptic inputs (26; 27). Presynaptic actions of AII have also been demonstrated in the nucleus tractus solitarius where Barnes et al (4) have demonstrated that excitatory effects of AII 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 AII 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 AII excites neurosecretory parvocellular neurons likely through a combination of activation of a putative NSCC and inhibition of I\(_k\). These findings are consistent with previous studies investigating the postsynaptic actions of AII on hypothalamic neurons. Such effects of AII in modulating specific ion channels which control the excitability of neuroendocrine PVN cells highlight cellular mechanisms through which AII may influence the secretion of anterior pituitary hormones.
Acknowledgements

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Figure Legends

Figure 1. Angiotensin II depolarizes neurosecretory parvocellular neurons. (A) Whole cell current clamp recordings illustrating the response of three neurosecretory parvocellular neurons to bath application of 0.01µM, 0.1µM, and 1.0µM AII (30s, application indicated by bar). Action potentials have been truncated. (B) Bar graph summarizing the responses of neurosecretory parvocellular neurons to AII application. 67% of neurosecretory parvocellular neurons responded to AII with a depolarization, while 5% hyperpolarized and 28% were unaffected. (C) Sigmoidal dose response curve shows that the depolarization of these neurons mediated by AII are dose dependent.

Figure 2. Angiotensin II mediated depolarization of neurosecretory parvocellular neurons is maintained in TTX and abolished by losartan. (A) Current clamp recording illustrating that the AII (0.1µM, 30s, application indicated by bar) induced depolarization of a neurosecretory parvocellular neuron is maintained following pretreatment with 1.0µM TTX (application indicated by light gray extended bar). Action potentials have been truncated. (B) Bar graph summarizes the effects of AII on neurosecretory parvocellular neurons following application of TTX. (C) Current clamp recording illustrating that the 0.1µM AII (30s, application indicated by bar) induced depolarization of a neurosecretory parvocellular neuron is significantly reduced by pretreatment with 1.0µM losartan (application indicated by white extended bar). Recording are made from the same neuron approximately 20 minutes apart. (D) Bar graph summarizes the effects of AII on neurosecretory parvocellular neurons following application of losartan.
Figure 3. Voltage-clamp recordings displaying currents recorded in response to slow voltage ramps following AII application. (A) Voltage-clamp recordings showing the currents produced by a 10 mV s\(^{-1}\) depolarizing ramps during ACSF, 0.1\(\mu\)M AII, and recovery following return to ACSF. The difference current obtained by subtracting control currents from currents measured during AII administration are displayed in the inset. (B) Average linear difference current obtained from the 7 cells (out of 12) that responded to 0.1\(\mu\)M AII during this slow ramp protocol. The reversal potential of this current was found to be -37.8±3.8mV.

Figure 4. Voltage-clamp recordings displaying a decrease in \(I_k\) following angiotensin II application. (A) Voltage-clamp step protocols (250 ms steps from -60 to +20 mV in the presence of TTX) revealed 0.1\(\mu\)M AII application results in a decrease in whole cell potassium currents. (B) \(I-V\) relationship of the 4 \(I_k\) cells that responded to 0.1\(\mu\)M AII (out of a total of 7 tested), illustrating that AII caused a significant decrease in this current in neurosecretory parvocellular PVN neurons in the -10 to +20 mV range (*\(P < 0.05\)).
Figure 1
Figure 2
Figure 3
Figure 4

A.

B.