ANG II-induced excitation of paraventricular nucleus magnocellular neurons: a role for glutamate interneurons

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Latchford, K. J., and A. V. Ferguson. ANG II-induced excitation of paraventricular nucleus magnocellular neurons: a role for glutamate interneurons. Am J Physiol Regul Integr Comp Physiol 286: R894–R902, 2004. First published January 15, 2004; 10.1152/ajpregu.00603.2003.—The hypothalamic paraventricular nucleus (PVN) plays a critical role in cardiovascular and neuroendocrine regulation. ANG II (ANG) acts throughout the periphery in the maintenance of fluid-electrolyte homeostasis and has also been demonstrated to act as a neurotransmitter in PVN exerting considerable influence on neuronal excitability in this nucleus. The mechanisms underlying the ANG-mediated excitation of PVN magnocellular neurons have yet to be determined. We have used whole cell patch-clamp techniques in hypothalamic slices to examine the effects of ANG on magnocellular neurons. Application of ANG resulted in a depolarization of magnocellular neurons, a response that was abolished in TTX, suggesting an indirect mechanism of action. Interestingly, ANG also increased the frequency of excitatory postsynaptic potentials/currents in magnocellular neurons, an effect that was abolished after application of the glutamate antagonist kynurenic acid. ANG was without effect on the amplitude of excitatory postsynaptic currents, suggesting a presynaptic action on an excitatory interneuron within PVN. The ANG-induced depolarization was shown to be sensitive to kynurenic acid, revealing the requisite role of glutamate in mediating the ANG-induced excitation of magnocellular neurons. These observations indicate that the ANGergic excitation of magnocellular PVN neurons is dependent on an increase in glutamatergic input and thus highlight the importance of a glutamate interneuron in mediating the effects of this neurotransmitter.

Electrophysiology; synaptic; neuroendocrine regulation

THE HYPOTHALAMIC PARAVENTRICULAR nucleus (PVN) plays major obligatory roles in autonomic and neuroendocrine regulation. Functional studies have demonstrated the involvement of PVN in the control of fluid electrolyte homeostasis, feeding behavior, cardiovascular regulation, stress adaptation, and the milk ejection reflex (11, 37). PVN is a heterogeneous nucleus whose neurons can be differentiated based on both morphological and electrophysiological criteria. Morphologically, PVN can be separated into the magnocellular neurons known to synthesize and secrete oxytocin (OXY) or vasopressin (AVP) in the circulation from terminals in the posterior pituitary, and the parvocellular neurons, which contain, among others, somatostatin, thyrotropin-releasing hormone, γ-aminobutyric acid (GABA), neurotensin, and corticotropin-releasing hormone (34, 37, 38). Electrophysiologically, PVN consists of type I (magnocellular: OXY/AVP) and type II (parvocellular) neurons, each of which has a unique electrical fingerprint (16, 28, 39). Interestingly, recent reports by Daftary et al. (6, 7) suggest the presence of a third previously unclassified glutamate interneuron in PVN thought to mediate noradrenergic (NA) input to PVN from the brain stem, although this group of neurons has yet to be characterized.

The actions of numerous peptides and hormones in PVN have been well documented. Recently, more attention has been paid to the synaptic regulation of magnocellular neurons along with the traditional membrane delimited mechanisms modulated by transmitters and other messengers. ANG II (ANG) has been shown to influence a variety of neuroendocrine and autonomic functions (5, 11, 12). ANG-containing cell bodies, nerve terminals, and receptors have been well documented in the PVN (20–22, 27, 32, 36). Early autoradiographic reports by Gehlert et al. (14, 15) and Mendelsohn et al. (29) demonstrated significant displaceable ANG binding in PVN, which after the development of antagonists specific for AT1 and AT2 receptors was shown to be AT1 specific. Functional studies then demonstrated that direct administration of ANG in PVN resulted in the excitation of neurosecretory cells identified as having projections to the posterior pituitary, increases in plasma AVP concentration, and an increase in blood pressure (9, 35). Furthermore, blockade of the AT1 receptor, by a specific antagonist (losartan), was shown to abolish these physiological actions of locally administered ANG (17) and of a postsynaptic effect of subfornical organ (SFO) stimulation-induced ANG release (25).

In vitro extracellular single unit recordings showed that bath application of ANG dose dependently increased the firing rate of neurons in PVN (24). This effect was maintained in synaptic isolation (low Ca2+/high Mg2+) and blocked by losartan, indicating the response was AT1 receptor mediated. Using whole cell voltage-clamp recordings, Li and Ferguson (26) later demonstrated that ANG inhibited the transient outward K+ current (IA), an effect that was also shown to be AT1 receptor mediated (26). However, there is to date no direct evidence explaining the specific mechanisms underlying the depolarizing effects of ANG on magnocellular neurons.

Recent biochemical reports demonstrating the apparent lack of AT1 receptors in the magnocellular region of PVN have provided an interesting and important caveat to these suggestions (21, 22). ANG receptors have been observed, however, in the paravascular regions of PVN, and Oldfield et al. (31) demonstrate that the AT1 receptor coexists strongly with neurons in the anterior paravascular area of the nucleus, which direct axons to the median eminence. Intriguingly, it has also been shown that, while ANG activates PVN magnocellular neurons, there is also a population of unidentified neurons that are influenced by activation of angiotensinergic pathways origi-
inating in SFO as well as by ANG itself (25). It is possible that these unidentified ANG-responsive neurons in PVN may be a subset of parvocellular interneurons within PVN, a suggestion supported by recent evidence suggesting the presence of a glutamatergic interneuron within PVN (2, 4, 6, 7).

We have utilized the whole cell patch-clamp technique to characterize the actions of ANG on magnocellular neurons in PVN slices. We report here that bath application of ANG results in the excitation of magnocellular neurons by both direct and synaptic mechanisms, the latter occurring as a consequence of enhanced glutamatergic input to these neurons.

**METHODS AND MATERIALS**

**Slice Preparation**

Experiments were performed using hypothalamic slices prepared as previously described (26). Briefly, Male Sprague-Dawley rats (150–250 g; 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 400 μM coronal sections were cut through the hypothalamus using a vibratome. Sections were hemisected, trimmed in blocks containing PVN, and incubated in oxygenated aCSF (95% O2-5% CO2) for at least 90 min at room temperature. Before being recorded, the slice was transferred to an interface-type recording chamber and continuously perfused with aCSF at a rate of 1 ml/min. All procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Queen’s University Animal Care Committee.

**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 (5,000 Hz) using the CED 1401 (CED, Cambridge, UK) 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. Excitatory postsynaptic potentials (EPSPs) were quantified based on frequency and amplitude (>1 mV) and shape (fast-rising phase and slow decay) using Spike2 software (CED). Similarly, excitatory postsynaptic currents (EPSCs) were quantified based on frequency and amplitude (>5 pA) and shape (fast-falling phase and slow decay). Each detected event was inspected visually to exclude obvious false EPSP/Cs. Mean group values were compared with a paired Student’s t-test. Dunnett’s multiple comparison test was used when multiple means were analyzed vs. a control group following a one-way ANOVA while a repeated-measures ANOVA and Newman-Keuls multiple comparison test were used when multiple means were analyzed.

**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 mosmol/l and pH between 7.3 and 7.4. The pipette solution contained (in mM) 140 potassium gluconate, 0.1 CaCl2, 2 MgCl2, 1.1 EGTA, 10 HEPES, and 2 NaATP and had a pH of 7.25 (adjusted with KOH if necessary). A stock solution of ANG (Phoenix Pharmaceuticals) was prepared from which daily aliquots were made to the required dilution. TTX (Alamone Laboratories) and kynurenic acid (KA; Tocris-Cookson) were prepared daily from stock solutions for those experiments where it was necessary to block voltage-activated Na+ channels or antagonize N-methyl-D-aspartate (NMDA) and non-NMDA receptors, respectively.

**RESULTS**

Whole cell patch-clamp recordings were obtained from a total of 60 PVN neurons that displayed a prominent “A current” after hyperpolarizing current pulses, had a linear current-voltage relationship, and therefore were classified as magnocellular neurons (Fig. 1A). These neurons had a mean resting membrane potential (RMP) of −57.7 ± 1.5 (SE) mV, displayed action potentials with a minimum spike amplitude of 60 mV, and had a mean input resistance of 1,100 ± 80 (SE) MΩ. Action potential and EPSP amplitude were measured throughout the duration of the recordings, and no rundown was observed.

**ANG Effects on Magnocellular Neurons**

Membrane potential. A total of 47 magnocellular neurons was tested for the effects of bath application of ANG in the current-clamp configuration. After a control recording period of at least 5 min, ANG was administered by bath perfusion in concentrations ranging from 0.01 to 10 μM for a period of 30 s. The majority of the neurons tested (n = 35, 74%) responded to ANG with a depolarization concurrent with an increase in action potential firing frequency, as illustrated in Fig. 1B and C. The remaining neurons tested with ANG either hyperpolarized (5%, n = 2) or were unaffected (21%, n = 10), showing no clear change in membrane potential (Fig. 1B). The depolarizing response of these magnocellular neurons to ANG was found to be dose dependent (10.0 μM: 11.8 ± 4.2 mV, n = 5; 1.0 μM: 9.1 ± 0.9 mV, n = 10; 0.1 μM: 6.9 ± 0.6 mV, n = 16; 0.01 μM: 1.4 ± 0.8 mV, n = 4) and demonstrated a sigmoidal dose-response relationship with an estimated EC50 of 5.6 × 10−8. In some cases after recovery of the neuron to resting RMP, ANG application was repeated (n = 4), and a second depolarization was observed, indicating that magnocellular neurons do not show significant desensitization to ANG. The depolarization observed in the magnocellular neurons was also accompanied by a change in input resistance, which was measured as the maximum voltage change to a hyperpolarizing current pulse. To eliminate the possibility that the depolarization of the neuron and subsequent activation of voltage-sensitive channels rather than ANG was responsible for the observed change in input resistance, current injection was used to hyperpolarize the membrane potential to RMP after the ANG-induced depolarization, and the input resistance was measured at this potential. Although relatively large changes in input resistance were observed, these effects were not consistent with five of eight cells tested, showing a decreased input resistance (∼410 ± 81 MΩ) in response to ANG while in three there was an increase (547 ± 80 MΩ). The mean change in input resistance for all of the neurons was not, however, significantly altered by ANG application (∼51.3 ± 183.5, P < 0.05, paired t-test).

Depolarizing effects of ANG on magnocellular neurons are significantly reduced in TTX. ANG effects on magnocellular neurons were also examined during sodium channel block with TTX (1.0 μM) to determine whether these were direct effects.
Figure 2A illustrates that the depolarizing effect of ANG (0.1 μM) was significantly reduced after bath application of TTX (ANG 0.1 μM: 7.0 ± 0.5 mV, TTX/ANG: 2.1 ± 0.6 mV, n = 6, P < 0.05, paired t-test). These data suggest that the action of ANG on magnocellular PVN neurons results from actions at a second subpopulation of PVN neurons, a surprising observation in view of previous voltage-clamp studies reporting that actions of \( I_A \) in magnocellular neurons are maintained in synaptic isolation (26). This observation led us to reexamine whether the effects of ANG on \( I_A \) in magnocellular neurons were observed in TTX. Indirect assessment of \( I_A \) was carried out using current-clamp techniques to measure the delay in return to baseline membrane potential after a standard hyperpolarizing current pulse (Fig. 2B). ANG inhibited \( I_A \), as measured by the return to baseline (control 1.1 ± 0.1 vs. ANG 0.5 ± 0.1 s, n = 11, P < 0.001, paired t-test), an effect that was maintained during bath perfusion with TTX (control 1.3 ± 0.1 vs. ANG/TTX 0.3 ± 0.1 s, n = 6, P < 0.001, paired t-test). \( I_A \) was further assessed by measuring the delay to first spike during a depolarizing current pulse from a hyperpolarized membrane potential. Such measurements also indicated ANG-induced reduction in \( I_A \) with a decrease in the time to first spike being observed as reported previously from our laboratory (1). These data are in agreement with previous voltage-clamp experiments showing ANG effects on \( I_A \) in magnocellular neurons in PVN (26) and supraoptic nucleus (SON) (30).
ANG increases EPSP/Cs in magnocellular neurons. Anatomic studies have confirmed the presence of glutamatergic interneurons within the PVN (4), whereas electrophysiological recordings have identified a physiological role for such intranuclear connections in mediating NA input to PVN from the brainstem (6, 7). Our unexpected observation that depolarizing effects of ANG were abolished in TTX led us to focus on the potential role of other synaptic inputs to magnocellular neurons in mediating these effects. We first carried out a retrospective analysis of excitatory postsynaptic potentials (EPSPs) in magnocellular neurons, which we had previously tested with ANG in these studies. Of these neurons, 63% (n = 1100/1722) were found to have spontaneous EPSPs. ANG application (0.1 μM, 30 s) resulted in a significant increase in EPSP frequency in a population of these magnocellular neurons (n = 10), as demonstrated in Fig. 3, A and B. The mean change in EPSP frequency is summarized in Fig. 3C (control: 2.2 ± 0.5 vs. ANG: 3.3 ± 0.6 Hz, n = 10, P < 0.005, paired t-test). In addition, when the EPSP frequency control data are normalized to 100% and compared with ANG, we observe a 62.4 ± 22.0% (n = 10, P < 0.05, t-test) increase in EPSP frequency. In the remaining magnocellular neurons (n = 12), we were unable to quantify an increase in EPSP frequency as a result of the commensurate increase in action potential frequency after depolarization. To eliminate the effects of action potential frequency or the change in membrane potential that would modify the amplitude of the observed events, these synaptic events were next analyzed in a separate population of magnocellular neurons using voltage-clamp techniques (Fig. 4). ANG was seen to cause significant increases in the frequency of EPSCs (control: 2.3 ± 0.5 vs. ANG: 3.7 ± 0.9 Hz, n = 6, P < 0.005, paired t-test). As with the EPSP data, when the EPSC frequency control data...
ANG-induced increase in synaptic activity is mediated by glutamate. To determine the neurotransmitter responsible for mediating the increase in synaptic events observed after ANG application on magnocellular neurons in PVN, additional experiments were performed using the nonspecific glutamate antagonist KA. Figure 5 clearly shows that pretreatment with KA (10 μM) completely abolished both spontaneous EPSCs ($n = 5$) and the increase in EPSC frequency in magnocellular neurons after ANG (0.1 μM, $n = 3$) administration. As predicted from previous studies (6, 13), such observations demonstrate that the EPSCs are glutamatergic and in addition provide supplementary support for the existence of a glutamate interneuron mediating the excitatory effects of ANG in PVN.

Depolarizing effects of ANG on magnocellular neurons are abolished by KA. The observations above raised the intriguing possibility that the ANG-induced depolarization of magnocellular neurons is mediated by glutamate and therefore should be abolished by glutamate antagonists. This hypothesis was tested in experiments where KA (10 μM) was bath applied before the application of ANG (0.1 μM) to examine the contribution of the increase in EPSPs to the observed depolarization (Fig. 6). Such pretreatment of slices abolished the increase in excitatory events and also significantly reduced the depolarization induced by ANG (ANG: $8.7 \pm 1.3$ mV vs. KA: $0.80 \pm 0.4$ mV, $n = 6, P < 0.001$, paired t-test, Fig. 6B). These observations support the requisite role of glutamatergic input to magnocellular neurons in their response to ANG.
DISCUSSION

Despite the numerous studies identifying physiological roles for ANG in PVN (for review, see Refs. 10–12 and 33), the cellular mechanisms through which this peptide depolarizes magnocellular neurons are still poorly understood. In the present study, using whole cell patch-clamp recording techniques, we show that, while the majority of magnocellular neurons do respond to ANG with dose-dependent depolarizations, these effects are abolished in TTX, suggesting they result from direct actions of ANG on a separate subpopulation of neurons in our slice preparation. Furthermore, we have demonstrated that such depolarizing drive to PVN magnocellular neurons appears to be the result of increased excitatory glutamatergic input, which presumably results from ANG actions increasing the activity of glutamate interneurons within the PVN.

Previous studies have reported that ANG inhibits the transient outward K⁺ current, $I_A$, of anatomically identified magnocellular neurons using whole cell voltage-clamp techniques to record from these neurons in hypothalamic brain slice preparations (26). Li and Ferguson (26) demonstrated that the effect of ANG on $I_A$ was maintained in low Ca²⁺, indicating that inhibition of this current was a direct effect of ANG on the magnocellular neurons rather than being synaptically mediated. Losartan, an AT₁ receptor antagonist, abolished the ANG inhibition of $I_A$, verifying the receptor-mediated nature of the response. Our current-clamp data support this conclusion by demonstrating that $I_A$ is inhibited in magnocellular neurons by
bath administration of ANG, an effect maintained in the presence of TTX. However, although this inhibition of I_A (which is known to play an important regulatory role in neuronal burst firing; see Ref. 3) may underlie the increases in spike frequency observed in magnocellular neurons after AT_1 receptor activation, it is unlikely to be responsible for the significant depolarizations observed in these neurons, which can even occur in quiescent cells.

The dissociation of these two effects is further emphasized by our unexpected observation that bath application of TTX, in contrast to its effects on I_A, significantly reduced the depolarizing response of the magnocellular neurons to ANG. The elimination of the ANG-mediated depolarization in magnocellular neurons would suggest that this action of ANG results from an indirect component that requires synaptic input. These data on the magnocellular neurons suggest that the ANG-induced depolarization of the magnocellular neurons is the result of this peptide’s actions on a secondary group of neurons either within or in the area immediately surrounding PVN (i.e., included in our slice preparation).

PVN is characteristically thought of as a heterogeneous nucleus composed of magnocellular and parvocellular neurosecretory neurons, and preautonomic neurons (37, 38). Historically, the existence of excitatory interneurons had been postulated, but only recently has this hypothesis been substantiated. The presence of short-latency EPSPs was discovered in the neurohypophysial system as early as 1973 (18). Ferguson et al. (8) later observed that a large population of PVN neurons (almost 40% of those recorded) exists that does not project to the posterior pituitary, median eminence, or dorsomedial medulla, again providing circumstantial evidence for interneurons within the PVN. Intriguingly, many of these cells were also excited by activation of SFO efferents, a synaptic effect that in some cases was abolished by losartan (25). Most recently, using radiolabeled aspartate as a neuronal marker for glutamatergic interneurons, Csaki et al. (4) provided histochemical data demonstrating that glutamatergic interneurons exist within PVN, and glutamatergic fibers that project to PVN are scattered throughout other hypothalamic nuclei and the telencephalon.

Boudaba et al. (2) provided the first evidence of the existence of excitatory synaptic circuits in PVN and SON, demonstrating that electrical or chemical stimulation of the dorsomedial hypothalamus or perifornical region resulted in evoked EPSPs in these nuclei. Daftary et al. (6, 7) later demonstrated that NA excitation of magnocellular neurons was effectively abolished by glutamate antagonists and TTX sensitive, suggesting this effect is mediated by an increase in glutamatergic input. In addition, they observed that the NA-mediated increase in EPSP frequency was maintained in a surgically isolated PVN preparation and also with NA microdrop application directly into PVN, indicating that the source of the EPSPs was a glutamatergic interneuron located within PVN. This suggestion gained recent support from histochemical studies demonstrating glutamatergic interneurons within PVN (4), although to date there are no reports in which chemical phenotyping of these cells has been combined with patch-clamp techniques to allow recordings to be obtained from this specific subset of PVN neurons.

We demonstrate here that these glutamatergic interneurons are also apparently responsible for the depolarization of the magnocellular neurons after ANG application. The observation that the ANG-induced depolarization in magnocellular neurons is TTX sensitive and therefore synaptically mediated suggested an alternate explanation for this peptide’s depolarizing effect on PVN magnocellular neurons. Interestingly, the majority of the magnocellular neurons that responded to ANG also displayed a significant increase in the frequency of EPSPs, suggesting glutamatergic input played an important role in the response of these neurons to ANG. However, we were unable to quantify an increase in EPSP frequency in several of the magnocellular neurons depolarized by ANG, since the large increase in action potential frequency concurrent with depolarization made it impossible to accurately count EPSPs, which in many cases likely drive these action potentials. We thus also evaluated the ANG effects using voltage-clamp techniques that avoid this problem and observed ANG-induced increases in EPSC frequency in the majority of magnocellular neurons tested. In addition, ANG was without effect on the amplitude of these EPSCs, suggesting that the effect of ANG is not the result of peptidergic effects on the nerve terminal and rather that this effect of ANG is likely the result of actions at the cell body of these glutamate interneurons. Subsequent experiments where the increase in EPSC frequency was abolished after application of the glutamate antagonist KA demonstrate the glutamatergic nature of these synaptic events. Finally, we tested our conclusion that glutamate inputs play an essential role in ANG signaling in PVN by examining the ability of
glutamate antagonists to influence the depolarizing effects of ANG in magnocellular neurons. As predicted, the depolarization of magnocellular neurons in response to ANG was found to be inhibited by application of KA, adding further support to the conclusion that glutamate interneurons play an essential prerequisite role in mediating ANG-induced depolarization of PVN magnocellular neurons.

Interestingly, Li et al. (23) demonstrated that ANG stimulates nonneurosecretory spinally projecting parvocellular PVN neurons by attenuating GABAergic synaptic inputs through activation and presynaptic AT1 receptors (23). Although the presynaptic disinhibition of parvocellular neurons by ANG is an intriguing observation, it does not seem a likely candidate to play a major role in modulating the excitation of magnocellular neurons via ANG. In fact, we have previously shown that ANG causes a nitric oxide-dependent increase in the frequency of inhibitory synaptic activity in magnocellular neurons, which appears to influence the excitability of these neurons (19).

Conclusions

The emergent notion of ANG’s action as a neurotransmitter in the central nervous system has led to a variety of studies aimed at characterizing its actions electrophysiologically. We
provide evidence here that ANG not only excites magnocellular neurons in PVN, a nucleus regarded for its critical role in neuroendocrine regulation, but does so via an increase in excitatory input to these neurons. These observations would also explain the excitation of magnocellular neurons by ANG in extracellular recordings given the apparent lack of AT1 receptors in mRNA in situ hybridization studies. Future studies should attempt to describe the ionic conductances that underlie the observed depolarization and a more detailed electrophysiological characterization of the glutamate interneuron and its response to ANG.

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