Chronotropic Effect of Angiotensin II via Type 2 Receptors in Rat Brain Neurons

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Zhu, Mingyan, Colin Sumners, Craig H. Gelband, and Philip Posner. Chronotropic effect of angiotensin II via type 2 receptors in rat brain neurons. J Neurophysiol 85: 2177–2183, 2001. Previously, we determined that angiotensin II (Ang II) elicits an Ang II type 2 (AT2) receptor–mediated increase of neuronal delayed rectifier K+ (I_KV) current in neuronal cultures from newborn rat hypothalamus and brain stem. This requires generation of lipoxygenase (LO) metabolites of arachidonic acid (AA) and activation of serine/threonine phosphatase type 2A (PP-2A). Enhancement of I_KV results in a decrease in net inward current during the action potential (AP) upstroke as well as shortening of the refractory period, which may lead to alterations in neuronal firing rate. Thus, in the present study, we used whole-cell current clamp recording methods to investigate the AT2 receptor–mediated effects of Ang II on the firing rate of cultured neurons from the hypothalamus and brain stem. At room temperature, these neurons exhibited spontaneous APs with an amplitude of 77.72 ± 2.7 mV (n = 20) and they fired at a frequency of 0.8 ± 0.1 Hz (n = 11). Most cells had a prolonged early after-depolarization that followed an initial shortening of the refractory period, which may lead to alterations in neuronal firing rate. Thus, in the present study, we used whole-cell current clamp recording methods to investigate the AT2 receptor–mediated effects of Ang II on the firing rate of cultured neurons from the hypothalamus and brain stem. At room temperature, these neurons exhibited spontaneous APs with an amplitude of 77.72 ± 2.7 mV (n = 20) and they fired at a frequency of 0.8 ± 0.1 Hz (n = 11). Most cells had a prolonged early after-depolarization that followed an initial fully developed AP. Superfusion of Ang II (100 nM) plus losartan (LOS, 1 μM) to block Ang II type 1 receptors elicited a significant chronotropic effect that was reversed by the AT2 receptor inhibitor PD 123,319 (1 μM). LOS alone had no effect on any of the parameters measured. The chronotropic effect of Ang II was reversed by the general LO inhibitor 5,8,11,14-eicosatetraenoic acid (10 μM) or by the selective PP-2A inhibitor okadaic acid (1 nM) and was mimicked by the 12-LO metabolite of AA 12-(S)-hydroxy-(5Z, 8Z, 10E, 14Z)-eicosatetraenoic acid. These data indicate that Ang II elicits an AT2 receptor–mediated increase in neuronal firing rate, an effect that involves generation of LO metabolites of AA and activation of PP-2A.

I N T R O D U C T I O N

Angiotensin II (Ang II) has been demonstrated to be an essential neuropeptide. It acts centrally to modulate blood pressure, baroreceptor reflexes, and fluid intake, effects that are mediated via the Ang II type 1 (AT1) receptors (Averill and Diz 2000; Culman et al. 1995; McKinley et al. 1996; Muratani et al. 1996). However, it is also apparent that the mammalian brain contains Ang II type 2 (AT2) receptors, sites that are abundantly expressed in neonates (Nuyt et al. 1999; Tsutsumi and Saavedra 1991) and that are not involved in mediating any of the well-known actions of Ang II. A number of studies have suggested that these brain AT2 receptors are involved in a variety of different physiological processes (Gallinat et al. 2000). For example, their abundance in neonates has led to the suggestion that AT2 receptors are involved in differentiation and development (Cook et al. 1991; Millan et al. 1991). These ideas are substantiated by studies that indicate a role for AT2 receptors in neurite outgrowth and cell migration (Cote et al. 1999; Laflamme et al. 1996), regeneration of optic nerve (Lucius et al. 1998), and apoptosis (Shenoy et al. 1999; Yamada et al. 1996). Investigations of mutant mice that lack the AT2 receptor gene have revealed that the knockout animals exhibit decreased exploratory behavior and spontaneous movements, increased basal blood pressure, an impaired drinking response to water deprivation, and lower basal body temperature (Hein et al. 1995; Ichiki and Inagami 1995a). More recent experiments from these mutant mice indicate a role for brain AT2 receptors in stress-induced hyperthermia (Watanabe et al. 1999). Thus, AT2 receptors are involved in the central control of certain behaviors and physiological responses. In addition, pathological roles for CNS AT2 receptors have been indicated by studies that show that global ischemia elicits a transient increase in AT2 receptor mRNA in rat brain (Makino et al. 1996) and that glutamate-induced toxicity of cultured cortical neurons is associated with increased expression of AT2 receptor mRNA and increased AT2 receptor binding (Shibata et al. 1998).

It is also apparent that stimulation of central neuronal AT2 receptors elicits effects at the fundamental level of changes in membrane ionic currents. For example, Ang II acts at AT2 receptors in non-differentiated NG108-15 neuroblastoma × glioma cells to inhibit T-type Ca2+ current (Buisson et al. 1995). Our previous studies of primary neurons cultured from newborn rat hypothalamus and brain stem showed that Ang II elicits increases both in voltage-dependent delayed rectifier K+ current (I_KV) and in A-type K+ current (I_A) (Kang et al. 1993). Furthermore, it is clear that the increase in I_KV is mediated through lipoxygenase (LO) metabolites of arachidonic acid (AA) and activation of serine/threonine phosphatase type 2A (PP-2A) (Kang et al. 1994; Zhu et al. 1998, 1999). The aim of the present set of studies was to determine whether the previously observed increases in neuronal K+ currents elicited by AT2 receptor stimulation result in a change in firing rate and, if so, what mechanisms are involved. Our data indicate that Ang II elicits an AT2 receptor–mediated increase in firing rate.
via a shortening of action potential (AP) duration. Furthermore, this chronotropic action of Ang II is mediated via activation of LO metabolites of AA and PP-2A.

**METHODS**

**Materials**

One-day-old Sprague-Dawley rats were obtained from our breeding colony, which originated from Charles River Farms (Wilmington, MA). Losartan potassium (LOS) was generously provided by Dr. William Henckler (Merck, Rahway, NJ). PD 123319 was purchased from Research Biochemicals International (Natick, MA); DMEM was obtained from Gibco (Grand Island, NY), crystallized trypsin (1 x) was from Cooper Biomedical (Malvern, PA), TTX was purchased from Calbiochem (La Jolla, CA), and plasma derived horse serum (PDHS) was obtained from Central BioMedia (Irwin, MO). Cytosine arabinoside (ARC), DNase 1, poly-L-lysine (MW 150,000), Ang II, ATP, guanosine 5’-triphosphate, CdCl2, HEPES, okadaic acid (OKA), 12-(S)-hydroxy-(5Z, 8Z, 10E, 14Z)-eicosatetraynoic acid [12-(S)-HETE], and 5,8,11,14-eicosatetraenoy acid (ETYA) were purchased from Sigma Chemical (St. Louis, MO). All other chemicals were purchased from Fisher scientific (St. Louis, MO).

**Preparation of neuronal cultures**

Neuronal co-cultures were prepared from hypothalami and brain stems taken from 1-day-old Sprague-Dawley rats, as described previously (Kang et al. 1993). Trypsin (375 U/ml)-and DNase 1 (496 U/ml)-disassociated cells were resuspended in DMEM containing 10% PDHS and were plated on 35-mm Nunc plastic tissue culture dishes pre-coated with poly-L-lysine. After the cells were grown for three days at 37°C in a humidified incubator with 95% O2 and 5% CO₂, they were exposed to 1 μM ARC for two days in fresh DMEM containing 10% PDHS. ARC was then removed and the cells were incubated with DMEM (10% PDHS) for an additional 9–12 days before use. At the time of use, cultures consisted of 90% neurons and 10% astrocyte glia, as determined by immunofluorescent staining with antibodies against neurofilament proteins and glial fibrillary acidic protein (Sumners et al. 1994). Neurons within these cultures contained AT1 and AT2 receptors, which are predominantly present in different cell populations (Gelband et al. 1997).

*Electrophysiological recordings*

Spontaneous and depolarizing pulse-elicited APs were recorded with the whole-cell voltage clamp configuration in current clamp mode (Hamill et al. 1981). Experiments were performed at room temperature (23–24°C) with an Axopatch 200B amplifier and a Digidata 1200B interface (Axon Instruments, Burlingame, CA). Data acquisition and analyses were performed with the use of Axoscope 7.0 and pClamp 6.2. Cells were bathed in Tyrode’s solution containing (in mM) 140 NaCl, 5.4 CaCl₂, 2.0 MgCl₂, 0.3 NaH₂PO₄, 10 HEPEs, and 10 dextrose, pH adjusted to 7.4 with NaOH. Neurons in the culture dish (volume 1.5 ml) were superfused at a rate of 2–4 ml/min. The patch electrodes (Kimax-5.1, Kimble Glass, Toledo, OH) had resistances of 3–4 MOhms when filled with an internal pipette solution containing (in mM) 140 KCl, 4 MgCl₂, 4 ATP, 0.1 guanosine 5’-triphosphate, 10 dextrose, and 10 HEPEs, pH adjusted to 7.2 with KOH. The whole-cell configuration was formed by applying negative pressure to the patch electrode. A junction potential of −8 mV was corrected for all membrane potentials. The resting membrane potential (RMP) was defined as the potential within a ±1 s time period during which there was no spontaneously firing AP. The neuronal firing rate was measured as the number of fully developed APs per second (Hz). The subthreshold activity was defined as a depolarization from the resting potential that did not fully develop into an AP. The early after-depolarization (EAD) was defined as a slow depolarization that immediately followed a fully developed AP. In some cases, a large spike depolarization was superimposed on an EAD: these spikes are referred to as EAD-APs (Figs. 2, 5, and 6). These EAD-APs varied widely in amplitude. Because an AP must reach a certain amplitude to fulfill its signal conduction role and, in our neuronal culture system, APs always had a peak depolarization beyond a membrane potential of 0 mV, we decided that the criteria for counting an EAD-AP as a fully developed AP would require a depolarization beyond 0 mV. Thus any EAD-AP that achieved this level of depolarization was counted in the firing rate; otherwise, it was only counted as an EAD. The AP amplitude was measured as the difference between the point of spike initiation and its peak amplitude. Because many of the neurons in culture at room temperature had a prolonged EAD, we only reported the time from the start of the AP to the time that the spike fell to half-amplitude (APD₅₀).

*Drug and antibody applications*

Ang II and drugs were dissolved in the appropriate solvent, followed by dilution in superfusate solution or patch pipette solution.
were followed by EADs, which resulted in low-amplitude APs from our previous studies (Wang et al. 1997), several of the APs in the cultures elicits a chronotropic effect (Wang et al. 1997). Therefore, in the present study, we aimed to assess the role of AT2 receptors in mediating these effects. Spontaneous APs recorded from neuronal cultures established in a given neuron. After this, 12-(S)-HETE (5 μl) was injected into the tip of the recording electrode via the PE-50 tube. From the pipette tip, the 12-(S)-HETE was allowed to diffuse into the neuron and measurements of firing rate were made 4 min later, at which time a stable peak response was obtained. Care was taken not to overperfuse the neuron, which was monitored electrically via the Axopatch and on a television monitor. Thus the concentrations of 12-(S)-HETE that are given in RESULTS refer to the amounts that were injected at the pipette tip and therefore are likely to be higher than the amounts that reached the site of action.

Data analysis

Results are expressed as means ± SE. Statistical significance was evaluated with paired Student’s t-test. Differences were considered significant at P < 0.05; n refers to the number of cells examined.

RESULTS

Selective stimulation of AT1 receptors in these neuronal cultures elicits a chronotropic effect (Wang et al. 1997). Because the aim of the present study was to assess AT2 receptor-mediated effects of Ang II on firing rate, recordings were performed in the presence of the AT1 receptor antagonist LOS (1 μM). Spontaneous APs recorded from neuronal cultures under these conditions are shown in Fig. 1. These data show spike potentials, EADs that do not produce a spike, and an EAD that does produce a spike (Fig. 1A). As was the case in our previous studies (Wang et al. 1997), several of the APs were followed by EADs, which resulted in low-amplitude APs (Fig. 1). Figure 1, B and C, shows an expansion of areas b and c in Fig. 1A and demonstrates the foot potential that precedes some spikes (B) as well as the EAD spike and the EAD sub-threshold potential (C). These APs are similar to those described previously by us and other investigators (Wang et al. 1997; Williams et al. 1996; Zhang and McBain 1995). The mean amplitude and time to 50% (APD50) repolarization of the APs recorded here were 77.72 ± 2.70 mV (n = 20) and 2.18 ± 0.08 ms (n = 9), respectively, in neurons treated with LOS alone. It is important to note that these parameters were not significantly different in the absence of LOS (mean amplitude 75.14 ± 3.54 mV, n = 7; APD50 2.16 ± 0.11 ms, n = 8). The apparent RMP of these neuronal cultures, in the presence of LOS, was −56.3 ± 3.7 mV (n = 22). The APs in the cells studied here fired at a rate ranging from 48 to 99 spikes per minute (SPM), with a mean of 72 ± 14 SPM, and the pattern was one of bursts and pauses (see Figs. 2, 5, and 7).

Superfusion of Ang II (100 nM) in the presence of LOS (1 μM) produced a rapid increase in firing rate, an effect that was inhibited by the AT2 receptor–selective ligand PD 123,319 (Fig. 2). PD 123,319 alone did not alter firing rate. Analysis of these data revealed that Ang II elicited a decrease in APD50, an
effect that was reversed by PD 123,319 (Fig. 3), which is consistent with the previously demonstrated stimulation of neuronal \( I_{KV} \). The neuronal cultures used here consist of a diverse population of cells. It was therefore possible that Ang II triggered the release of a neurotransmitter from one of the neurons in the dish, which resulted in a paracrine effect at the neuron from which recordings were being made. To investigate this possibility, neurons were superfused with CdCl\(_2\) (0.3 mM) prior to the application of Ang II to prevent neurotransmitter release. Under these conditions, the Ang II (100 nM)-induced increase in firing rate (Fig. 4) and the decrease in APD\(_{50}\) (control 2.14 ± 0.07 ms; Ang II 1.88 ± 0.06 ms; Ang II/CdCl\(_2\) 1.91 ± 0.05 ms; \( n = 3 \) neurons) were not altered. These data suggest a direct action of Ang II at the neuron from which recordings were being made. In addition, inclusion of CdCl\(_2\) (0.3 mM) within the superfusate did not alter basal firing frequency, which indicates that the APs and EADs are not based on a calcium current (Fig. 4). The data presented in Fig. 4 also indicate that all APs and EADs are eliminated by the presence of TTX (1.5 \( \mu M \)) within the superfusate, which suggests that they are based on an inward sodium current.

Because the positive chronotropic effect of Ang II was mediated by the \( \text{AT}_2 \) receptor, we proceeded to study the intracellular mechanisms through which this response was transduced. Our previous studies indicated that the stimulatory action of Ang II, via \( \text{AT}_2 \) receptors, on neuronal \( I_{KV} \) involved the generation of 12-lipoxygenase (12-LO) metabolites of AA and activation of PP-2A (Kang et al. 1994; Zhu et al. 1998, 2000). We therefore decided to test the roles of 12-LO metabolites of AA and PP-2A in the positive chronotropic action of Ang II. Superfusion of cultured neurons with ETYA (10 \( \mu M \)), a general LO inhibitor, produced no changes in baseline firing rate. However, the presence of ETYA within the superfusate completely abolished the Ang II–induced increase in firing rate (Fig. 5) and decrease in APD\(_{50}\) (control 2.26 ± 0.09 ms; Ang II 2.01 ± 0.08 ms; Ang II/ETYA 2.19 ± 0.1 ms; \( n = 5 \) neurons), which indicates a role for LO metabolites of AA in this effect. Furthermore, intracellular application of 12-(S)-HETE (1 \( \mu M \)), a 12-LO metabolite of AA whose receptors are primarily cytosolic (Herbertsson et al. 1999), produced an increase in firing rate similar to that obtained with Ang II via the \( \text{AT}_2 \) receptor (Fig. 6). 12-(S)-HETE (1 \( \mu M \)) also elicited a
decrease in APD$_{50}$ (control, 2.16 ± 0.05 ms; 12-(S)-HETE, 2.01 ± 0.07 ms; $n$ = 3 neurons). Superfusion of neuronal cultures with OKA (1 nM), a selective inhibitor of PP-2A, did not affect spontaneous firing rate by itself (Fig. 7). However, OKA completely inhibited the positive chronotropic effect (Fig. 7) and reduction in APD$_{50}$ (control, 2.23 ± 0.04 ms; Ang II, 2.03 ± 0.09 ms; Ang II/OKA, 2.23 ± 0.13; $n$ = 5 neurons) elicited by 100 nM Ang II, which indicates a role for PP-2A in this response.

**DISCUSSION**

Behavior and homeostatic regulation are modulated by alterations in neuronal activity via changes in AP firing rates and firing patterns. One of the physiological modulators of these behaviors has been shown to be the octapeptide Ang II. This neuropeptide works via an interaction with different receptor subtypes to modulate such functions as catecholamine, nitric oxide (NO), bradykinin, and PGF$_2\alpha$ release as well as blood pressure, fluid homeostasis, apoptosis, cell growth, and neurite outgrowth (Cook et al. 1991; Cote et al. 1999; Gallinat et al. 2000; Hein et al. 1995; Ichiki and Inagami 1995a; Lafllamme et al. 1996; Millan et al. 1991; Shenoy et al. 1999; Yamada et al. 1996). The AT$_2$ receptor has been implicated as a modulator of apoptosis, neurite development, and exploratory behavior (Hein et al. 1995; Ichiki and Inagami 1995b; Okuyama et al. 1999; Shenoy et al. 1999) and recent studies suggest that it plays a role in hypertension via NO and PGF$_2\alpha$ (Carey et al. 2000a). In addition, a number of studies indicate that the AT$_2$

**FIG. 6.** 12-(S)-hydroxy-(5Z, 8Z, 10E, 14Z)-eicosatetraynoic acid [12-(S)-HETE] exerts a positive chronotropic action on neuronal cultures. **A** and **B**: recordings of APs from a representative neuron under various conditions. **A**: intracellular application of pipette solution. **B**: intracellular application of 12-(S)-HETE (1 $\mu$M). C: means ± SE from 4 neurons showing the chronotropic effect of 12-(S)-HETE expressed as percent change of frequency. *, $P < 0.001$ compared with control recordings.

**FIG. 7.** The positive chronotropic effect of AngII is abolished by the selective serine/threonine phosphatase type 2A inhibitor okadaic acid (OKA). **A**–**C**: recordings of APs made from a representative neuron under various conditions. **A**: superfusion of the AT$_1$ receptor antagonist LOS (1 $\mu$M) alone. **B**: superfusion of Ang II (100 nM) in the presence of LOS (1 $\mu$M). **C**: superfusion of Ang II (100 nM) in the presence of LOS (1 $\mu$M) and OKA (1 nM). **D**: means ± SE from 4 neurons showing the chronotropic effect of Ang II in each treatment situation. *, $P < 0.05$ compared with control recordings (LOS alone). OKA (1 nM) alone had no significant effects on firing rate.
receptor has a modulatory role in those actions triggered by stimulation of AT1 receptors (Carey et al. 2000b; Gelband et al. 1997). As a model of the in vivo situations just described, various groups used brain slices and cultured neonatal neurons to study the cellular mechanisms through which Ang II elicits actions via the AT2 receptor (Ambuhl et al. 1992; Kang et al. 1993; Li and Ferguson 1993; Xiong and Marshall 1994; Zhu et al. 1998). In fact, Li and Ferguson (1993) found that both AT1 and AT2 receptor activity could increase firing rate in a population of neurons in rat brain slices.

Our group has recorded from single neurons isolated from newborn rat hypothalamus and brain stem in culture to study the signal transduction pathways through which Ang II increases $I_A$ and $I_{KV}$ after binding to the AT2 receptor. The increase in these currents would lead both to an increased rate of repolarization as well as to a shortened AP refractory period in single neurons (Zhang and McBain 1995). This shortened refractory period is reflected in the AP as a reduction of APD$_{50}$. In the present study, we showed that Ang II does in fact exert a positive chronotropic effect in the cultured neurons studied, an effect mediated via AT2 receptors. The increased firing rate in the presence of Ang II appears to be caused, in large part, by bursts of APs that occur after an AP that has a shortened APD$_{50}$ but that does not become fully repolarized (Figs. 2, 5, and 7). Because $I_A$ inactivates more rapidly than $I_{KV}$, after the initial summation of these currents there would be a period of reduced outward current. This may result in a net inward current being able to trigger an AP earlier than it could under conditions where Ang II is absent. This trigger might be similar to that seen in some cardiac pacemaker cells (DiFrancesco 1981; Yanagihara and Irisawa 1980). However, another possibility is that Ang II activates a receptor-dependent depolarizing inward current that may underlie the increase in firing rate. This will be part of our future investigations.

Because of the mixed population of neuronal cells in the cultures used, it was important to demonstrate that the chronotropic effect is directly mediated through the AT2 receptor located on the cell studied. This was accomplished by blocking synaptic release of neurotransmitter with Cd prior to and during the application of Ang II. As can be seen in Fig. 4, the chronotropic effect persisted in the presence of blocked synaptic release.

We had shown previously that the AT2 receptor-mediated increase in $I_{KV}$ could be substantially but not completely blocked by interfering with the production of AA and its 12-LO metabolites (Zhu et al. 1998, 2000). In addition, this Ang II–induced stimulation of $I_{KV}$ was totally blocked by OKA in a concentration that selectively blocks PP-2A (Kang et al. 1994). We had further demonstrated, through the use of single-cell RT-PCR, that all of the components for this transduction pathway were localized in the cell that had been studied electrophysiologically (Zhu et al. 1998). In the present study, we showed that ETYA, a blocker of the LO pathway, can substantially reduce the positive chronotropic effect of Ang II (Fig. 5) whereas intracellular application of 12-(S)-HETE (a 12-LO metabolite of AA) induces a positive chronotropic effect (Fig. 6). Furthermore, we demonstrated that the use of OKA to block the activation of PP-2A can totally block the positive chronotropic effect of Ang II (Fig. 7). These results indicate that the intracellular signaling pathways involved in AT2 receptor–mediated chronotropic action are similar to those involved in the stimulation of $I_{KV}$ by Ang II. In addition, the present data confirm the role of $I_{KV}$ in the positive chronotropic action of Ang II mediated through AT2 receptors.

Based on our previous studies, which demonstrated that Ang II increases $I_{KV}$ and $I_A$ via the AT2 receptor (Kang et al. 1993), we propose that the positive chronotropic effect reported here is a result of the shortened refractory period (Fig. 3) caused by an enhancement of $I_{KV}$ and $I_A$ as well as the depolarization caused by an inward current following the termination of $I_A$. How this positive chronotropic mechanism, mediated through the AT2 receptor, translates into physiological function remains to be demonstrated. However, based on the potential roles of the AT2 receptor in behavior, volume regulation, and apoptosis (Gallinat et al. 2000), it may be speculated that the increased firing rate in certain neuronal pathways will act to affect specific behaviors, modulate the effects mediated by AT1 receptors, and, in individual neurons exposed to ischemic conditions, lead to calcium loading and, potentially, apoptosis (Makino et al. 1996; Yu et al. 1997, 1999).

These findings relating to changes in spontaneous firing rate and signal transduction are consistent with those demonstrated in various other models including single cells, brain slices, in vivo recording, and whole animals, and supply a cellular level explanation of the mechanisms underlying the various effects resulting from the activation of AT2 receptors by Ang II.

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AT$_2$ RECEPTORS AND NEURONAL FIRING RATE


