Chronotropic Action of Angiotensin II in Neurons via Protein Kinase C and CaMKII

Chengwen Sun, Colin Sumners, Mohan K. Raizada

Abstract—Angiotensin II (Ang II) plays an important role in the central control of blood pressure and baroreflexes. These effects are initiated by stimulation of Ang II type 1 (AT₁) receptors on neurons within the hypothalamus and brain stem, and involve increasing the activity of noradrenergic, substance P, and glutamatergic pathways. The goal of this study is to investigate the intracellular signaling molecules, which are involved in mediating the Ang II-induced increases in neuronal activity. Using neurons in primary culture from newborn rat hypothalamus and brain stem, we have previously determined that Ang II elicits an AT₁ receptor-mediated inhibition of delayed rectifier K⁺ current, a stimulation of Ca²⁺ current, and a consequent increase in firing rate. In the present study we have demonstrated that this chronotropic action of Ang II in neuronal cultures involves activation of Ca²⁺-dependent signaling molecules. The Ang II-induced increase in firing rate was abolished by inhibition of phospholipase C with U73122 (10 μmol/L), and was attenuated by the protein kinase C inhibitor calphostin C (10 μmol/L) or by the calcium/calmodulin-dependent kinase II (CaMKII) inhibitor KN-93 (10 μmol/L). A combination of calphostin C and KN-93 completely inhibited this Ang II action. These results indicate that the AT₁ receptor-mediated increase in neuronal firing rate involves activation of both PKC and CaMKII, and suggest that these enzymes are potential targets for manipulating the central actions of Ang II. (Hypertension. 2002;39[part 2]:562-566.)

Key Words: angiotensin II □ receptors, angiotensin □ protein kinases □ rats □ nervous system □ neuropeptides

It is established that the brain angiotensin system exerts regulatory influences in the control of blood pressure and plays an important role in the development and establishment of hypertension. 1,2,3,4 Accumulating evidence points to angiotensin II (Ang II) and other derivatives of the central angiotensin system as possible neurotransmitters or neuromodulators in specific pathways that connect major cardiovascular and autonomic regulatory centers in the brain stem, hypothalamus, and forebrain. 5,6 Stimulation of angiotensin II type 1 (AT₁) receptors elicits increases in blood pressure, arginine vasopressin release, salt appetite, and drinking behavior, 1,2,7 effects that participate in the regulatory role of this peptide on extracellular fluid volume and cardiovascular hemodynamics. These physiological effects of Ang II are associated with an increase in neuronal firing rate and firing pattern produced by activation of AT₁ receptors located in the hypothalamus and brain stem. 8,9,10 However, the underlying intracellular mechanisms that are involved in this chronotropic action of Ang II in the brain are not well established. An understanding of these mechanisms is crucial since they are responsible for mediating changes in neuronal activity induced by Ang II, and will ultimately contribute to the alterations in cardiovascular hemodynamics induced by this peptide.

We approached this problem in previous studies using neurons cultured from newborn rat hypothalamus and brain stem. Activation of AT₁ receptors by Ang II in these neurons inhibits delayed rectifier K⁺ current (Iₓ), and A-type K⁺ current (Iₐ), and stimulates total Ca²⁺ current. 11 The changes in Iₓ, Iₐ, and total Ca²⁺ current are consistent with the chronotropic action of Ang II in these cells, mediated by AT₁ receptors. 12 Analysis of the intracellular pathways that are involved in the modulation of neuronal K⁺ and Ca²⁺ currents by Ang II reveals the involvement of phospholipase C (PLC) and Ca²⁺-dependent signaling molecules including protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaMKII). 11,13 Changes in neuronal activity or firing rate are controlled through alterations in action potential (AP) frequency or duration, which in turn depend on the activity of membrane K⁺ and Ca²⁺ currents. Thus, we propose that the chronotropic action of Ang II in neuronal cultures is mediated through activation of PKC and CaMKII. The data obtained in the present study indicate that the AT₁ receptor-mediated increase in neuronal firing rate involves PLC-dependent increases in PKC and CaMKII activation, thus supporting our proposal.

Methods

Materials
One-day-old Wistar-Kyoto (WKY) or Sprague-Dawley (SD) rats were obtained from our breeding colony, which originated from...
Charles River Farms (Wilmington, Mass). Losartan potassium (LOS) was generously provided by Dr. William Henckler, Merck and Co. (Rahway, NJ). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from GIBCO. Crystallized trypsin (1X) was from Cooper Biomedical. Plasma-derived horse serum (PDHS), cytosine arabinoside (ARC), DNase I, poly-l-lysine (MW 150 000), PD123319, calphostin C, KN-93, KN-92, U73122, and all other chemicals were purchased from Sigma-Aldrich Chemicals.

**Preparation of Neuronal Cultures**

Neuronal cultures were prepared from the hypothalamus and brain stem of newborn WKY or SD rats exactly as described previously. 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. Unless noted, experiments were performed using WKY rat cultures.

**Electrophysiological Recordings**

Spontaneous- and depolarizing-pulse-elicited action potentials (APs) in neuronal cultures were recorded with the use of the whole cell voltage clamp configuration in current clamp mode, exactly as described previously.

**Data Analysis**

Results are expressed as means ± SE. Statistical significance was evaluated with the use of a 1-way ANOVA followed by a Newman-Keuls test. Differences were considered significant at P<0.05.

**Results**

**AT1 Receptor-Mediated Increase in Firing Rate**

Spontaneous APs recorded from WKY rat neurons in primary culture displayed electrophysiological properties similar to those described previously by other investigators and in our own studies using neurons from SD rats. The mean amplitude and time to 50% (APD50) repolarization of the APs recorded were 71.63±4.18 mV (n=12) and 2.26±0.09 ms (n=12), respectively. These properties were not significantly altered by the AT1 receptor antagonist PD123319 (1 μmol/L) (mean amplitude, 71.24±8.1 mV, n=20; APD50, 2.21±0.10 ms, n=12). This is important because the selective stimulation of Ang II type 2 (AT2) receptors in neurons in primary culture elicits a chronotropic effect. Since the goal of this study was to assess the AT1 receptor-mediated effects of Ang II on neuronal firing rate, all subsequent recordings were performed in the presence of 1 μmol/L PD123319. The apparent resting membrane potential (RMP) of these neurons, in the presence of PD123319, was −51.2 mV ±3.5 mV (n=42). The APs in the neurons studied here fired at a rate ranging from 20 to 91 spikes per minute (SPM) with a mean of 61±25 SPM (n=42), and the pattern was one of random burst firing. Superfusion of Ang II (1 μmol/L) in the presence of PD123319 (1 μmol/L) produced a rapid increase in firing rate from a control level of 0.4±0.1 Hz to 1.4±0.5 Hz (n=12). This action of Ang II was completely reversed by the AT1 receptor-selective antagonist Losartan (LOS, Figure 1). LOS alone did not alter firing rate (data not shown). This indicates that Ang II has a positive chronotropic effect on neuronal firing rate via AT1 receptors in neurons cultured from rat hypothalamus and brain stem.

**Ang II-Induced Increase in Firing Rate Involves PLC**

Ang II exerts a positive chronotropic action on neuronal cultures via the AT1 receptor. A to C, Recordings of APs from a representative neuron under the following conditions: A, superfusion of the AT2 receptor antagonist PD123319 (PD; 1 μmol/L); B, superfusion of Ang II (100 nmol/L) in the presence of PD (1 μmol/L); and C, superfusion of Ang II (100 nmol/L) in the presence of PD (1 μmol/L) and the AT1 receptor inhibitor LOS (1 μmol/L). D, Bar graphs are means±SE from 12 neurons showing the chronotropic effect of Ang II in each treatment situation. *P<0.05 compared with control recordings (LOS alone). PD (1 μmol/L) alone had no significant effects on firing rate.

**Ang II-Induced Increase in Firing Rate Involves PKC and CaMKII**

In this series of experiments we investigated the role of PKC and CaMKII in the AT1 receptor-mediated increase in neuronal firing rate. Protocols similar to those used in the above PLC experiments were followed. Ang II (100 nmol/L), superfused in the presence of 1 μmol/L PD123319, triggered a significant increase in neuronal firing rate (Figure 3). This chronotropic action of Ang II was significantly reduced, but not abolished, by the selective PKC inhibitor calphostin C (Cal, 10 μmol/L; Figure 3). Attenuation of the Ang II-induced increase in firing rate by Cal was also observed in SD rat neuron cultures, demonstrating similar effects in cells from another normotensive rat strain (C. Sumners, unpublished data). The increase in firing rate elicited by Ang II (100 nmol/L) via AT1 receptors was also attenuated, but not

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**Figure 1. Ang II exerts a positive chronotropic action on neuronal cultures via the AT1 receptor. A to C, Recordings of APs from a representative neuron under the following conditions: A, superfusion of the AT2 receptor antagonist PD123319 (PD; 1 μmol/L); B, superfusion of Ang II (100 nmol/L) in the presence of PD (1 μmol/L); and C, superfusion of Ang II (100 nmol/L) in the presence of PD (1 μmol/L) and the AT1 receptor inhibitor LOS (1 μmol/L). D, Bar graphs are means±SE from 12 neurons showing the chronotropic effect of Ang II in each treatment situation. *P<0.05 compared with control recordings (LOS alone). PD (1 μmol/L) alone had no significant effects on firing rate.**
completely blocked, by superfusion of the selective CaMKII inhibitor KN-93 (10 μmol/L; Figure 4). In contrast, superfusion of 10 μmol/L KN-92, an inactive analog of KN-93, did not alter the Ang II-induced increase in firing rate (data not shown). Cal did not significantly alter basal firing rate, nor did KN-93 or KN-92 (Figures 3 and 4). These results suggest that activation of both PKC and CaMKII is required for the Ang II-induced chronotropic effect. This was confirmed by experiments in which combined superfusion of Cal and KN-93 completely abolished the Ang II-induced increase in neuronal firing rate (Figure 5).

Discussion

The precise firing pattern of neuronal APs depends on both synaptic inputs and their intrinsic membrane properties. The latter are governed by alterations in membrane ionic currents through direct receptor-ion channel interactions or through receptor-mediated changes in intracellular messengers. Pre-

Figure 2. The positive chronotropic effect of Ang II is abolished by the PLC inhibitor U73122. A to D, Recordings of APs made from a representative neuron under the following conditions: A, superfusion of the AT2 receptor antagonist PD123319 (PD; 1 μmol/L) alone; B, superfusion of Ang II (100 nmol/L) in the presence of PD (1 μmol/L); C, following washout of Ang II and PD, superfusion of PD and U73122 (10 μmol/L); and D, superfusion of Ang II (100 nmol/L) in the presence of PD (1 μmol/L) and U73122 (10 μmol/L). E, Bar graphs are means ± SE from 11 neurons showing the chronotropic effect of Ang II in each treatment situation. *P < 0.05 compared with control recordings (PD123319 alone). U73122 (10 μmol/L) alone had no significant effects on firing rate.

Figure 3. The positive chronotropic effect of Ang II is attenuated by the PKC inhibitor calphostin C. A to D, Recordings of APs made from a representative neuron under the following conditions: A, superfusion of the AT2 receptor antagonist PD123319 (PD; 1 μmol/L) alone; B, superfusion of Ang II (100 nmol/L) in the presence of PD (1 μmol/L); C, following washout of Ang II and PD, superfusion of PD (1 μmol/L) and calphostin C (Cal; 10 μmol/L); and D, superfusion of Ang II (100 nmol/L) in the presence of PD (1 μmol/L) and Cal (10 μmol/L). E, Bar graphs are means ± SE from 7 neurons showing the chronotropic effect of Ang II in each treatment situation. *P < 0.05 compared with control recordings (PD123319 alone). Cal alone had no significant effects on firing rate.

Figure 4. The positive chronotropic effect of Ang II is reduced by the selective CaMKII inhibitor KN-93. A to D, Recordings of APs made from a representative neuron under the following conditions: A, superfusion of the AT2 receptor antagonist PD123319 (PD; 1 μmol/L) alone; B, superfusion of Ang II (100 nmol/L) in the presence of PD (1 μmol/L); C, following washout of Ang II and PD, superfusion of PD (1 μmol/L) and KN-93 (10 μmol/L); and D, superfusion of Ang II (100 nmol/L) in the presence of PD (1 μmol/L) and KN-93 (10 μmol/L). E, Bar graphs are means ± SE from 7 neurons showing the chronotropic effect of Ang II in each treatment situation. *P < 0.05 compared with control recordings (PD123319 alone). KN-93 alone had no significant effects on firing rate.

Figure 5. The positive chronotropic effect of Ang II is completely abolished by coinhibition of PKC and CaMKII. APs were recorded using the same protocols as in Figures 3 and 4, except that neurons were cotreated with Cal (10 μmol/L) and KN-93 (10 μmol/L). The data shown are means ± SE from 5 neurons in each treatment situation. *P < 0.05 compared with control recording (PD123319 alone). Treatment with a combination of KN-93 and Cal alone had no effects on firing rate.
viously, we determined that Ang II increases PKC and CaMKII activities in neuronal cultures, and that the Ang II-induced changes in neuronal K⁺ and Ca²⁺ currents involve activation of PLC and Ca²⁺-dependent intracellular signaling pathways.⁸,¹¹,¹² Consistent with these findings, our present data demonstrate for the first time that the chronotropic action of Ang II via AT₁ receptors involves PLC and subsequent activation of PKC and CaMKII. However, the question remains as to whether the Ang II-induced modulation of K⁺ currents and Ca²⁺ currents via PKC and CaMKII is causally linked with the increases in neuronal firing rate produced by Ang II. Support for such an association comes from our previous studies, which show that inhibition of either I_K or I_Na using tetraethylammonium (TEA) and 4-aminopyridine, respectively, mimics the effects of Ang II and causes an increase in basal firing rate.¹² In addition, in the same studies we determined that inhibition of Ca²⁺ currents with Cd²⁺ elicits an inhibition of firing rate. This proves that inhibition of K⁺ currents and stimulation of Ca²⁺ currents in our neuronal system cause a chronotropic action. In preliminary experiments we have determined that Ang II (100 nmol/L in the presence of 1 μmol/L PD123319 to block AT2 receptors) does not enhance or potentiate the increased firing rate produced by superfusion of 3 mmol/L TEA (Chengwen Sun et al, unpublished observations, 2002). Collectively, these findings suggest that the effects of Ang II on currents and firing rate are causally linked, but this relationship will only be established through further investigations.

The involvement of both PKC and CaMKII in the modulation of neuronal activity by Ang II has certain functional implications. For example, it is possible that each kinase provides a unique regulatory influence on I_K, which leads to differential effects on neuronal activity and ultimately cellular/whole animal functions. Such influences may take the form of distinct actions on the biophysical properties of the Kv2.2 channel, which mediates the inhibitory effects of Ang II on neuronal I_K.⁴ Thus, PKC and CaMKII may have unique regulatory influences on channel properties such as activation, inactivation, open or closed time, or time to first latency. Once we have a better understanding of the mechanisms by which PKC and CaMKII regulate Kv2.2, we will be able to determine whether there are distinct actions of each kinase on channel activity and ultimately neuronal firing rate. A further implication from these data is that factors that regulate the activity of PKCα or CaMKII independent of Ang II will influence the AT₁ receptor-mediated inhibition of I_K and firing rate. For example, a factor that inhibits CaMKII activity may blunt the chronotropic actions of Ang II.

A major question from these studies concerns the biochemical mechanisms through which PKC and CaMKII elicit modulation of neuronal activity. It is well known that K⁺ channel proteins contain consensus phosphorylation sequences for PKC, CaMKII, and tyrosine kinases,⁵ and a major mechanism of regulation of channel activity is via phosphorylation/dephosphorylation at these sites.⁶ Inspection of the amino acid sequence of Kv2.2 reveals the presence of a number of consensus PKC and CaMKII phosphorylation sites within the cytosolic domains of the channel protein.⁷ Thus, one possibility is that PKC and CaMKII inhibit neuronal I_K, and ultimately the chronotropic action of Ang II, by direct phosphorylation of the Kv2.2 channel proteins. Our preliminary experiments indicate that Ang II does increase the phosphorylation of Kv2.2 (Sumners et al, unpublished data). However, a role for PKC and CaMKII, and details of the specific phosphorylation sites have yet to be established. It is also important to point out that other mechanisms of modulation of K⁺ channel activity exist. These include phosphorylation of cytoskeletal proteins, which in turn regulate channel activity by causing conformational changes in channel subunits⁸ or regulation mediated through interactions of G protein subunits, kinases, and channel proteins within lipid rafts.⁹ Neither of these mechanisms can be excluded in terms of Ang II-induced modulation of Kv2.2 (I_K) and neuronal activity.

The physiological role of the positive chronotropic effect of Ang II must also be further investigated. We determined previously that AT₁ receptor stimulation in neuronal cultures increases the release of norepinephrine.³⁰ In addition, it is clear that Ang II increases release of Substance P in the medulla.³¹ Thus, it is tempting to speculate that the positive chronotropic effect of Ang II, via PKC and CaMKII, may contribute to neurotransmitter release. This idea will be the subject of future investigations.

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**References**

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