PI3-Kinase Inhibitors Abolish the Enhanced Chronotropic Effects of Angiotensin II in Spontaneously Hypertensive Rat Brain Neurons

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Sun, Chengwen, Jianqing Du, Colin Sumners, and Mohan K. Raizada. PI3-kinase inhibitors abolish the enhanced chronotropic effects of angiotensin II in spontaneously hypertensive rat brain neurons. J Neurophysiol 90: 3155–3160, 2003. First published August 6, 2003; 10.1152/jn.00222.2003. Angiotensin II (Ang II), acting at Ang II type 1 receptors (AT1Rs), increases the firing rate of neurons from Wistar-Kyoto (WKY) rat brain via protein kinase C (PKC)- and calcium-calmodulin kinase II (CaMKII)-dependent mechanisms. The objectives of this study were twofold; first, to compare the Ang-II-stimulated increase in firing of neurons from WKY and spontaneous hypertensive rats (SHR) and second, to elucidate the signaling mechanisms involved. Action potentials were measured in neurons cultured from SHR and WKY rat brains using the whole cell configuration of the patch-clamp technique in the current-clamp mode. Ang II (100 nM) caused three- and sixfold increases in neuronal firing rate in WKY rat and SHR neurons, respectively; effects that were abolished by the AT1R antagonist Losartan (1 μM). Co-administration of calphostin C (10 μM, a PKC inhibitor) and KN-93 (10 μM, a CaMKII inhibitor) completely blocked this Ang II action in WKY rat neurons, while they caused only a ~50% attenuation in SHR neurons. The residual increase in firing rate produced by Ang II in SHR neurons was blocked by inhibitors of phosphatidylinositol 3 kinase (PI3-kinase), either LY 294002 (10 μM) or wortmannin (100 nM). These observations suggest that a PI3-kinase signaling pathway may be responsible for the enhanced chronotropic effect produced by Ang II in SHR neurons.

INTRODUCTION

It is well established that the brain angiotensin system exerts regulatory influences in the control of blood pressure (BP) and thus plays a key role in the development and establishment of hypertension (Averill and Diz 2000; de Gasparo et al. 2000; Muratani et al. 1996). The cardiovascular actions of angiotensin II (Ang II) within the brain involve increases in vasopressin release, dampening of baroreflexes and stimulation of sympathetic pathways via activation of angiotensin type 1 receptors (AT1Rs) in cardioregulatory hypothalamic and brain stem nuclei (Averill et al. 2000; Casto and Phillips 1986; Ferguson et al. 2001; McKinley et al. 1996; Muratani et al. 1996; Paton et al. 2001). The significance of the brain Ang II system in BP control and hypertension is further supported by studies with the spontaneously hypertensive rat (SHR). These rats exhibit increased expression and activity of AT1Rs in a number of hypothalamic and brain stem regions such as the paraventricular nucleus, median preoptic nucleus, subformical organ, nucleus tractus solitarius, and dorsal motor nucleus of the vagus (Gehlert et al. 1986; Gutkind et al. 1988; Han and Sim 1998; Lebrun et al. 1996; Lu et al. 1994; Raizada et al. 1999; Song et al. 1994). In addition, interruption of brain AT1R function by pharmacological or genetic means lowers BP in the SHR (Lu et al. 1994). Despite these well-documented enhanced physiological actions of Ang II via brain AT1Rs in the SHR, the cellular and molecular basis of these effects remain poorly understood. We have developed an in vitro neuronal cell culture model from prehypertensive SHR to investigate the mechanisms of AT1R-mediated increases in Ang II actions in the SHR brain. Studies from this model have established that increases in AT1R levels and Ang-II-induced norepinephrine neuromodulation observed in the adult SHR brain are preserved in vitro in neuronal cultures from prehypertensive rat brain. This has led us to propose that a hyperactive brain angiotensin system in the SHR is genetically linked and is not the result of an increase in BP (Lebrun et al. 1996).

It is clear that the physiological response elicited by Ang II acting in the brain involves modulation of specific neuronal pathways (Raizada et al. 1999). This modulation includes rapid changes in neuronal activity, and our group has investigated the underlying cellular and intracellular events. These studies indicate that Ang II inhibits both delayed rectifier K+ current (I\textsubscript{Ks}) and transient (A type) K+ current (I\textsubscript{A}) and stimulates total Ca\textsuperscript{2+} current via the AT1R (Raizada et al. 1999; Sumners et al. 2002). These effects are consistent with our observation that Ang II elicits a positive chronotropic action involving PKC and CaMKII signaling pathways (Wang et al. 1997; Zhu et al. 1997).

Our objectives in the present study were twofold: to investigate whether the effects of Ang II on neuronal firing rate are altered in SHR versus WKY rats and to delineate the signaling pathways linking the AT1R to neuronal activity in neurons from both strains. The rationale for these objectives was based on our previous observations that SHR neurons express higher levels of AT1R and exhibit increased norepinephrine neuromodulation compared with WKY rat neurons (Lu et al. 1994).
METHODS

Materials and animals

Twelve-week-old male and female WKY and SHR rats were obtained from Charles River Farms (Wilmington, MA). These rats were used as breeders to produce a constant supply of newborn SHR and WKY rat pups. The mean blood pressures of the SHR and WKY rat breeders were 166 ± 2 and 117 ± 5 mm Hg, respectively. Rats were housed at 25 ± 2°C on a 12 h:12 h light-dark cycle and provided with food and water ad libitum. All animal protocols were approved by the University of Florida Animal Care and Use Committee.

Losartan potassium (Los) was generously provided by Merck (Rahway, NJ). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Gibco (Grand Island, NY). KN-93 and calphostin C were purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). Ang II, plasma-derived horse serum (PDHS), and other chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO).

Preparation of neuronal cultures

Neuronal co-cultures were prepared from the hypothalamus and brain stem of newborn SHR and WKY rats exactly as described previously (Sumners et al. 1990). Trypsin (375 U/ml) and DNase I (496 U/ml) dissociated brain cells were suspended in DMEM containing 10% PDHS and were plated in poly-l-lysine-precocated 35-mm-diam tissue culture dishes at 3.0 × 10^5 cells/dish. Cultures were grown in a humidified incubator at 37°C for 12–15 days prior to use. At the time of use, cultures consisted of 90% neurons and 10% astroglia as determined by immunofluorescent staining with antibodies against neurofilament proteins and glial fibrillary acidic protein (Summers et al. 1990). Neurons within these cultures contained both AT1 and Ang II type 2 receptors (AT2R) on distinct neuronal cells (Summers et al. 2002).

Electrophysiological recordings

Spontaneous action potentials (APs) were recorded from WKY and SHR neurons at room temperature using the patch-clamp technique in current-clamp mode. Recordings were taken from a morphologically distinct population of multipolar neurons, and of these, 65% responded to Ang II. The recordings were made using an Axon Digidata 1200 B interface (Axon Instruments, Burlingame, CA) as described previously (Zhu et al. 2001). Briefly, cells were bathed in Tyrode’s solution containing (in mM) 140 NaCl, 5.4 KCl, 2 MgSO4, 2 CaCl2, 0.3 NaH2PO4, 10 dextrose, and 10 HEPES, pH 7.4. Neurons were superfused at a rate of 2–4 ml/min. The patch electrodes have resistances of 2–4 MΩ when filled with an internal pipette solution containing (in mM) 140 KCl, 4 MgCl2, 10 dextrose, 10 HEPES, 4 ATP, and 0.1 GTP, pH 7.2. The whole cell configuration was obtained by applying negative pressure to the patch electrode. The resting membrane potential (RMP) was defined as the potential within a 1-s time period during which there was no spontaneous APs. The RMP for control WKY rat neurons was −57.44 ± 3.78 mV (n = 7) and that for control SHR neurons was −57.99 ± 2.98 mV (n = 7). These values were not altered by Ang II (100 nM) treatment, i.e., −56.36 ± 3.49 mV (n = 7) in WKY rat neurons and −56.70 ± 2.68 mV (n = 7) in SHR neurons. The neuronal firing rate was measured as the number of fully developed APs per second (Hz) essentially as described previously (Zhu et al. 2001). Ang II treatment elicited bursting firing, and so the data from Ang II-treated neurons represents the average of both intraburst firing and burst firing.

Data analysis

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

RESULTS

Chronotropic effects of Ang II in SHR and WKY rat neurons

Spontaneous APs recorded from either SHR or WKY rat neurons exhibited similar electrophysiological properties. In addition, these properties were similar to those observed in Sprague-Dawley (SD) rat neurons, another normotensive control (Lebrun et al. 1996). For example, the mean amplitude and time to 50% repolarization (APD50) of the APs recorded were 75.1 ± 5.3 mV (n = 11) and 2.31 ± 0.18 ms (n = 11), respectively, in WKY rat neurons. In SHR neurons, the mean amplitude and APD50 of the APs displayed were 74.8 ± 4.4 mV (n = 9) and 2.72 ± 0.21 ms (n = 9), respectively. In addition, the basal firing rate of WKY rat neurons was 0.43 ± 0.07 Hz (n = 10) compared with 0.49 ± 0.06 Hz in SHR neurons (n = 10). The neuronal cultures used in the present study also contain AT2R, and because their selective stimulation elicits a chronotropic action (Zhu et al. 2001), thus all experiments in the present study were performed in the presence of the AT2R blocker, PD123,319 (1 μM). This treatment with PD123319 did not alter basal firing rate (Fig. 1).

Spontaneous APs were recorded in the presence of PD123,319, and there was no significant difference in the firing rate between SHR and WKY rat neurons (Fig. 1). Superfusion of Ang II in the presence of PD123,319 (1 μM) elicited increases in firing rate in both WKY rat and SHR neurons, effects that were concentration-dependent. For example, in WKY rat neurons, superfusion of 10 and 100 nM Ang II produced increases in firing rate of 97 ± 17% (n = 4) and 324 ± 65% (n = 10), respectively. By contrast, in SHR neurons, the effects of Ang II were significantly greater. Superfusion of 10 and 100 nM Ang II produced respective increases in firing rate of 268 ± 45% (n = 4) and 556 ± 64% (n = 10). The data for 100 nM Ang II are presented in Fig. 1. Furthermore, the chronotropic actions of Ang II in both rat strains were abolished by 1 μM losartan, an AT1R selective antagonist (Fig. 1). Losartan alone did not alter the firing rate of SHR or WKY rat neurons (data not shown).

Effects of PKC and CaMKII inhibitors on the chronotropic response to Ang II in SHR and WKY rat neurons

Our recent studies have demonstrated that the chronotropic effects of Ang II via AT1, R are mediated by activation of PKC and CaMKII in neurons from normotensive (SD and WKY) rats (Sun et al. 2002). Here, we have used a pharmacological strategy to determine whether PKC- and CaMKII-dependent mechanisms are also responsible for the chronotropic response produced by Ang II in SHR neurons. Figure 2 shows that Ang II (100 nM) in the presence of 1 μM PD123,319 increased the firing rate of SHR and WKY rat neurons. As expected, this effect was significantly greater in the neurons from SHR. Combined superfusion of the PKC inhibitor calphostin C (10 μM) and the CaMKII inhibitor KN-93 (10 μM) did not alter the basal firing rate of SHR or WKY rat neurons (Fig. 2). However, these agents completely abolished the chronotropic effect of Ang II in WKY rat neurons. In contrast, the increase in firing rate produced by Ang II in SHR neurons was attenuated by −50%, but not abolished, after preincubation of cultures with calphostin C (10 μM) and KN-93 (10 μM; Fig. 2). Superfusion of neuronal cultures with DMSO (1:1,000), the
solvent for calphostin C and KN-93, produced no such reduction in the Ang-II-induced increase in firing (data not shown). These data suggested that a PKC- and CaMKII-independent intracellular signal transduction pathway may account for the enhanced chronotropic response to Ang II in SHR neurons.

Effect of PI3-kinase inhibitors on the chronotropic effects of Ang II

Our previous studies have established that Ang II stimulates PI3-kinase in both WKY and SHR neurons (Yang et al. 1996; Yang and Raizada, 1999). Although the role of PI3-kinase in the modulation of neuronal activity is not known, it mediates the neurotogenic effect of Ang II in WKY rat neurons (Yang et al. 2002b). These observations led us to hypothesize that the chronotropic action of Ang II in SHR neurons that is not inhibited by calphostin C, and KN-93 may involve PI3-kinase. Thus the role of PI3-kinase in the Ang-II-induced increase in firing rate in SHR and WKY rat neurons was examined using the PI3-kinase inhibitors, LY294002 (10 μM) and wortmannin (100 nM). Figure 3 demonstrates that superfusion of SHR and WKY rat neuronal cultures with Ang II (100 nM) in the presence of 1 μM PD123,319 produced the expected increases in firing rate and that superfusion of these cells with 10 μM LY294002 did not significantly alter basal firing rate. The data also demonstrate that although LY 294002 did not affect the Ang-II-induced chronotropic effect in WKY rat neurons, it significantly blunted this Ang II action in SHR neurons (Fig. 3). In fact this PI3-kinase inhibitor reduced the Ang-II-induced chronotropic effect in SHR neurons to the level seen in WKY rat neurons (data not shown).

Ang-II-induced increases in firing rate in SHR neurons are abolished by co-incubation with CaMKII-, PKC-, and PI3-kinase inhibitors

In the next series of experiments, we examined the effect of simultaneous incubation of neurons with PI3-kinase, PKC, and

**FIG. 1.** Angiotensin I (Ang II) produces an Ang II type 1 receptor (AT1R)-mediated increase in firing rate in spontaneous hypertensive rat (SHR) and Wistar-Kyoto (WKY) rat neurons. A–C: representative tracings showing the action potentials (APs) recorded from a single WKY rat neuron under the following sequential treatment conditions. A: superfusion of 1 μM PD123,319 (PD) for 5 min, followed by superfusion of Ang II (100 nM, B) in the presence of PD for 5 min; This was followed by superfusion of Ang II in the presence of PD and 1 μM Losartan (Los, C). D–F: representative tracings showing the APs recorded from a single SHR neuron under the same sequential treatment conditions as in A–C. G: bar graphs are means ± SE showing the chronotropic effect of Ang II on WKY rat neurons and SHR neurons in each treatment situation. Numbers in parentheses indicate the number of neurons per group. *, P < 0.01 compared with the respective control recording. #, P < 0.01 compared with PD + Ang II in WKY neurons.

**FIG. 2.** Effect of protein kinase C (PKC)- and calcium-calmodulin kinase II (CaMKII) inhibitors on the chronotropic action of Ang II in SHR and WKY rat neurons. Bar graphs are means ± SE showing the chronotropic effects of Ang II in SHR and WKY rat neurons under the following sequential treatment conditions. Neurons were first superfused with 1 μM PD for 5 min, followed by superfusion of Ang II (100 nM) in the presence of PD for 5 min. After washout of Ang II, neurons were exposed to a combination of PD plus 10 μM calphostin C (Cal) and 10 μM KN-93 for 5 min. This was followed by superfusion of Ang II in the presence of the same drug combination. Firing rate was recorded under each treatment situation, and data are presented as the increase in firing as a percent of control (100%). Numbers in parentheses are number of neurons in each case. *P < 0.05 compared with the respective control recordings (PD alone) in each group. #P < 0.05 compared with PD + Ang II in WKY neurons.
CaMKII inhibitors on the chronotropic effect induced by Ang II in SHR neurons. Figure 4 shows that Ang II (100 nM) triggered a significant increase in firing rate in SHR neurons. This effect was completely abolished by the combined superfusion of calphostin C (10 μM), KN-93 (10 μM), and LY294002 (10 μM). This further suggests that PI3-kinase-dependent signaling may be responsible for the enhanced chronotropic response to this peptide in SHR neurons.

DISCUSSION

The most significant finding in the present study is that the enhanced chronotropic effect of Ang II in SHR neurons is blocked by inhibition of PI3 kinase. This suggests that PI3 kinase may be exclusively involved in the chronotropic actions of Ang II. The diagram in Fig. 5 summarizes our hypothesis, suggesting that Ang II acts at AT1R in SHR and WKY rat neurons to produce an increase in firing rate via signaling pathway that involves parallel activation of PKC and CaMKII (Pan et al. 2001; Sumners et al. 2002). In addition, AT1R in SHR neurons may be linked to a PI3 kinase-signaling pathway that is responsible for the enhanced chronotropic response to Ang II in SHR. This hypothesis is primarily based on the use of pharmacological agents to inhibit these kinases, and we must use some caution in interpretation of this data. Further evidence with the use of PI3 kinase dominant negative mutants will be needed to confirm these ideas.

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**FIG. 3.** Effect of phosphatidylinositol 3 kinase (PI3 kinase inhibitors on the Ang-II-induced chronotropic effect in SHR and WKY rat neurons. A–D: representative tracings showing the APs recorded from a single WKY rat neuron that was superfused sequentially as follows. PD (1 μM) for 5 min (A), followed by Ang II (100 nM) plus PD for 5 min (B), followed by a 5-min wash. Next, the neuron was superfused with PD plus 10 μM LY294002 (LY) for 5 min (C), followed by Ang II in the presence of PD and LY for 5 min (D). E–H: representative tracings showing the APs recorded from a single SHR neuron under the same conditions as in A–D. I: bar graphs are means ± SE showing the firing rate under each condition. Data are presented as the change in firing as a percent of control (100%), and numbers in parentheses are the number of neurons. *, P < 0.05 compared with the respective control recordings (PD alone). #, P < 0.05 compared with PD + Ang II in WKY neurons.

**FIG. 4.** The Ang-II-induced chronotropic effect in SHR neurons is abolished by co-incubation with inhibitors of PKC, CaMKII, and PI3-kinase. A–D: representative tracings showing the APs recorded from a single SHR neuron that was superfused sequentially as follows. PD (1 μM) for 5 min (A), then Ang II (100 nM) plus PD (1 μM, B), followed by superfusion with a combination of PD (1 μM), KN-93 (KN, 10 μM), calphostin C (Cal, 10 μM), and LY294002 (LY, 10 μM, C) for 5 min. Last, the neuron was superfused with Ang II in the presence of PD, LY, KN, and Cal for 5 min (D). E: bar graphs are means ± SE showing the firing rate recorded under each treatment condition. Data are presented as the change in firing as a percent of control (100%). *P < 0.01 compared with control. Cal, KN, and LY alone had no significant effects on firing rate.
Our previous studies have established that the expression of AT₁R is two- to fourfold higher in neuronal cultures for SHR hypothalamus/brain stem compared with equivalent cultures from normotensive (WKY, and SD) rats (Frame and Cohen 2001; Gehlert et al. 1986). Consistent with this is a parallel increase in Ang II’s transcriptionally regulated neuromodulatory effects in SHR neurons, involving a Ras-MAP kinase signaling pathway (Lu et al. 1998; Yang et al. 1996). These and other studies have established that the additional increase of AT₁R-mediated neuromodulation is linked to the genetics of hypertension rather than being a consequence of the increase in BP because the neurons are prepared from prehypertensive SHR (Raizada et al. 1999). The present study suggests that an additional but distinct signaling system involving PI3 kinase may be relevant for the enhanced chronotropic response to Ang II in the SHR. It also raises a number of important questions concerning the mechanism of PI3-kinase action. First, are the pharmacological agents selectively targeting PI3 kinase? This is relevant in view of a potential lack of specificity of these agents. Use of dominant negative mutants of PI3 kinase would be of significance answering this issue. Second, what is the role of PI3-kinase in normotensive rat brain neurons because Ang II stimulates this enzyme in both WKY and SHR? We believe, based on evidence, that PI3-kinase activation is linked to Ang-II-induced neuritogenesis in WKY rat neurons (Yang et al. 2002). Its activation by Ang II in the SHR is linked to neuronal activity. This hypothesis predicts that the signaling downstream from PI3 kinase must be distinct to provide this diversity in these two strains of neurons. There is no evidence currently in favor or against this hypothesis, but the possibility cannot be discounted. Third, what is the downstream signaling mechanism for PI3-kinase-mediated simulation of neuronal firing rate in the SHR? PI3-kinase activation results in many cellular effects that are mediated by glycogen synthesis kinase-3 (GSK-3) (Eldar-Finkelman et al. 2002; Frame and Cohen 2001). It appears that GSK-3 is not involved in the regulation of neuronal activity. This view is based on our preliminary data indicating that inhibition of GSK-3 by 3-(4-carboxy-4-chloroanilino)-4-(3-nitrophenyl) maleimide had no effect on the Ang-II-induced increase in neuronal activity in the SHR. This leads us to hypothesize that PI3 kinase may directly modulate the activity of ion channels or channel-associated proteins that are involved in the regulation of neuronal activity. There is indirect evidence to support this view. For example, the Ang-II-induced regulation of calcium channel activity in vascular smooth muscle cells involves direct actions of PI3 kinase (Macrez et al. 2001; Northcott et al. 2002). In addition, PI3-kinase-mediated activation of calcium channels in neurons has also been observed (Blair et al. 1997). However, we cannot exclude the possibility that the effects of PI3 kinase on neuronal activity in the SHR neurons are indirect. For example, it is well known that PI3 kinase activates atypical PKCs in a number of tissues (e.g., Takeda et al. 1999). Thus the stimulation of neuronal firing by PI3 kinase in the SHR may be indirect and involve activation of an atypical PKC.

The concept that PI3-kinase signaling is unique in SHR neurons and could be linked to a hyperactive brain angiotensin system has evolved from our in vitro experiments. However, this view is also supported by in vivo studies. For example, Ang II stimulates PI3-kinase activity in the hypothalamus and brain stem, two cardio-regulatory relevant brain areas (Yang et al. 1999). In addition the inhibition of PI3 kinase in the rostral ventrolateral medulla of the brain stem, a site at which Ang II increases neuronal excitation (Li and Guyenet 1995), decreases both basal- and Ang-II-induced increases in BP exclusively in the SHR in vivo (Seyedabadi et al. 2001). Last, gene-profiling data indicate a significant decrease in the expression of the regulatory subunit (p85) of PI3 kinase in the SHR brain (Yang et al. 2002). This decrease would lead to an increase in the activity of the catalytic subunit of PI3-kinase. In spite of this evidence, we believe that further in vitro and in vivo studies must be performed to validate the relevance of a proposed hyperactivity of brain PI3 kinase in BP control in SHR. Nonetheless, these observations suggest that inhibitors of PI3-kinase alter SHR brain neuronal activity and that the enzyme could be an important therapeutic target for the control of neurogenic hypertension.

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DIS CLO SURES

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REFERENCES

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DISCLOSURES

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