Angiotensin Selectively Activates a Subpopulation of Postganglionic Sympathetic Neurons in Mice

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Abstract—Angiotensin II (Ang II) increases renal sympathetic nerve activity in anesthetized mice before and after ganglionic blockade, suggesting that Ang II may directly activate postganglionic sympathetic neurons. The present study directly tested this hypothesis in vitro. Neurons were dissociated from aortic-renal and celiac ganglia of C57BL/6J mice. Cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) was measured with ratio imaging using fura 2. Ang II increased [Ca\(^{2+}\)]\(_i\), in a subpopulation of sympathetic neurons. At a concentration of 200 nmol/L, 14 (67%) of 21 neurons responded with a rise in [Ca\(^{2+}\)]\(_i\). The Ang II type 1 (AT\(_1\)) receptor blocker (losartan, 2 μmol/L) but not the Ang II type 2 (AT\(_2\)) receptor blocker (PD123,319, 4 μmol/L) blocked this effect. The Ang II–induced [Ca\(^{2+}\)]\(_i\), increase was abolished by removal of extracellular Ca\(^{2+}\) but not altered by depletion of intracellular Ca\(^{2+}\) stores with thapsigargin. Ang II no longer elicited a [Ca\(^{2+}\)]\(_i\) increase in the presence of lanthanum (25 μmol/L). The specific N-type and L-type Ca\(^{2+}\) channel blockers, ω-conotoxin GVIA and nifedipine, respectively, significantly inhibited the Ang II–induced [Ca\(^{2+}\)]\(_i\), increase. The protein kinase C inhibitor H7 but not the protein kinase A inhibitor H89 blocked the response to Ang II. These results demonstrate that Ang II selectively activates a subpopulation of postganglionic sympathetic neurons in aortic-renal and celiac ganglia, triggering Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels. This effect is mediated through AT\(_1\) receptors and requires the activation of protein kinase C. The activation of a subgroup of sympathetic neurons by Ang II may exert unique effects on kidney function in pathological states associated with elevated Ang II. (Circ Res. 2001; 88:787-793.)

Key Words: calcium imaging ■ calcium influx ■ calcium channel blockers ■ protein kinase activation

The renin-angiotensin system plays an important role in the regulation of arterial blood pressure and body fluid and electrolyte homeostasis. Angiotensin II (Ang II) directly contracts vascular smooth muscle cells and enhances the renal tubular reabsorption of sodium.\(^1,2\) In addition, Ang II contracts vascular smooth muscle cells and enhances the sympathetic nervous system activity.\(^3,4\) Mounting evidence supports a role of the sympathetic nervous system in the long-term regulation of arterial pressure.\(^5-8\) Ang II has been shown to modulate the sympathetic nervous system function through actions within the central nervous system (eg, at the area postrema and rostral ventrolateral medulla) and through facilitation of sympathetic ganglionic neurotransmission and neurotransmitter release from efferent sympathetic terminals.\(^9\)

We recently described biphasic effects of Ang II on renal sympathetic nerve activity (RSNA) in intact mice.\(^10\) Intravenous administration of Ang II increased arterial pressure and evoked a biphasic change in RSNA: inhibition of high-amplitude phasic bursts of RSNA secondary to the initial rise of arterial pressure followed by the activation of low-amplitude continuously discharging RSNA. The initial inhibition of RSNA was prevented by baroreceptor denervation, indicating that it was indirectly mediated by the baroreflex response to the rise in arterial pressure. Consistent with this interpretation, the ganglionic blocker hexamethonium eliminated the baseline high-amplitude phasic bursts of RSNA. However, neither denervation nor hexamethonium prevented the Ang II–induced activation of low-amplitude continuously discharging RSNA, suggesting a direct action of Ang II on postganglionic sympathetic neurons. The goal of the present study was to test this hypothesis in vitro by directly measuring Ang II–induced changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in cultured sympathetic neurons isolated from mouse aortic-renal (ARG) and celiac (CG) ganglia.

Materials and Methods

Animals
All experiments were performed on cells obtained from C57BL/6J mice (Harlan; Madison, Wis). The project had been approved by the University of Iowa Animal Care and Use Committee.

Cell Culture
Primary cultures of sympathetic neurons were prepared from the ARG and CG of mice. Adult C57BL/6J mice (25 to 30 g) were
anesthetized with sodium pentobarbital (60 mg/kg IP). The left kidney was exposed through a left flank incision, and the left RGA and CG were isolated from the connective tissue and excised for dissociation and cell culture as described previously.11,12 The ganglia were incubated in the digestive medium for 55 minutes at 37°C. Enzymatic activity was terminated by a trypsin inhibitor. After centrifugation, the cells were resuspended in modified L-15 medium with 1% BSA and 0.02% pluronic solution. After removal of the loading medium, the cells were rinsed twice and kept in normal extracellular solution and then loaded with the Ca²⁺-sensitive indicator fura 2-AM at a concentration of 2 μmol/L, along with 1% BSA and 0.02% pluronic solution. After removal of the loading medium, the cells were rinsed twice and kept in normal extracellular solution at room temperature for at least 30 minutes to allow deesterification of fura 2-AM. The coverslip was mounted in a recording chamber on an inverted fluorescence microscope. A 75-W xenon lamp served as the light source. The light passed through interference filters mounted on a computer-controlled shutter, allowing subsequent imaging at excitation wavelengths of 340 nm and 380 nm. The emitted light passed through a filter set (wavelength 510 nm) and was measured with a CCD camera (Photon Technology International). All images were automatically corrected for background fluorescence. The camera data were collected every 4 seconds, digitized, and stored online on a personal computer (Photon Technology International). With this configuration, 1 to 3 neurons were usually studied within a given field. The ARG and CG neurons were distinguished from glial cells by their distinct morphology and size. The isolated sympathetic neurons were round with a large soma (∼20 μm in diameter) and did not possess processes after 24 hours in culture. Glial cells typically measured <10 μm and had an elongated spindle-shaped appearance. Moreover, glia did not respond to potassium-induced depolarization with an increase in [Ca²⁺], confirming their identity. Regions of interest were selected visually for the data analysis. Typically, the loading was homogeneous and did not allow identification of subcellular compartments. In some cells, the indicator appeared to be compartmentalized in certain areas of the cell, as indicated by a fluorescence ratio that clearly differed from the ratio measured in other areas of the cytoplasm. These regions were excluded from further analysis. The images obtained at 340 nm and 380 nm were divided pixel by pixel to generate the ratio values. In consideration of the spatial resolution of videofluoroscopy, the apparent [Ca²⁺], is a composite of values from the free cytosolic Ca²⁺ and the Ca²⁺ compartmentalized in organelles.14 Therefore, we decided to use only ratio values to express changes in the fluorescent signals. An increase in the ratio value corresponds to an increase in [Ca²⁺]. A significant increase in [Ca²⁺] was defined as an increase of at least 10% above baseline. All experiments were performed at room temperature.

**Experimental Protocols**

**Effects of Ang II on [Ca²⁺] in Sympathetic Neurons**

To determine whether Ang II altered the Ca²⁺ homeostasis in sympathetic neurons, we applied Ang II to the bath solution to reach a desired final concentration (50 to 500 nmol/L). Neurons that responded with a minimum of 10% increase from baseline were considered responsive.

**Roles of AT₁ and AT₂ Receptors in Mediating Response of [Ca²⁺] to Ang II**

To identify the receptor subtype that mediates the Ang II–induced [Ca²⁺] increase, we pretreated the neurons with the selective Ang II type 1 (AT₁) and Ang II type 2 (AT₂) receptor antagonists losartan (2 μmol/L) and PD123,319 (4 μmol/L), respectively.

**Role of Ca²⁺ Influx Versus Ca²⁺ Release From Intracellular Ca²⁺ Stores**

A rise in [Ca²⁺], can be due to Ca²⁺ influx from the extracellular compartment or release from intracellular Ca²⁺ stores. To determine whether Ca²⁺ influx is required for the Ang II–induced [Ca²⁺], increase, experiments were performed in Ca²⁺-free solution. Conversely, we depleted intracellular Ca²⁺ stores to test the role of Ca²⁺ release by pretreating neurons with Ca²⁺-ATPase inhibitor thapsigargin (500 nmol/L) in Ca²⁺-free solution for 10 minutes. To confirm that this protocol completely depleted intracellular Ca²⁺ stores, we stimulated neurons with 10 mmol/L caffeine or reapplied thapsigargin after washout of the initial thapsigargin in Ca²⁺-free solution.

**Role of Voltage-Sensitive Ca²⁺ Channels**

To determine the role of voltage-sensitive Ca²⁺ channels in the Ang II–induced [Ca²⁺], increase in sympathetic neurons, we performed experiments after pretreatment with a nonselective Ca²⁺ channel blocker lanthanum [La³⁺] (25 μmol/L) and the specific N-, L-, and P/Q-type Ca²⁺ channel blockers ω-conotoxin GVIA (2 μmol/L), nifedipine (10 μmol/L), and ω-agatoxin IVA (1 μmol/L), respectively.

**Role of Protein Kinase Activation in Ang II–Induced Increase in [Ca²⁺]**

To assess whether protein kinase activation is involved in Ang II–induced intracellular signal processing, we used an experimental design that assesses Ca²⁺ influx in response to the addition of extracellular Ca²⁺ after depletion of intracellular Ca²⁺ stores with thapsigargin (500 nmol/L). The neurons were pretreated with inhibitors of protein kinase A (PKA) (H89, 1 μmol/L) and protein kinase C (PKC) (H7, 10 μmol/L).

**Data Analysis**

Group data are expressed as mean ± SE. The paired t test was used to analyze responses to a single intervention. For protocols involving multiple comparisons, the data were analyzed by ANOVA, followed by the Newman-Keuls post hoc test. The statistical analysis was performed by using GB-STAT 6.0 software (Dynamic Microsystems, Inc). A value of P<0.05 was considered to indicate statistical significance.

**Chemicals and Solutions**

Unless indicated otherwise, the normal extracellular solution contained 140 mmol/L NaCl, 5.4 mmol/L KCl, 1 mmol/L MgCl₂, 2 mmol/L CaCl₂, and 5.6 mmol/L glucose, buffered with HEPES and NaOH to pH 7.4 (osmolality 300 mOsm). For experiments with Ca²⁺-free solution, we omitted Ca²⁺ and added 0.5 mmol/L EGTA. Under those conditions, 3 mmol/L MgCl₂ was used to avoid significant differences in the surface-charging effect. Fura 2-AM and pluronic solution were obtained from Molecular Probes; ω-agatoxin IVA was obtained from Peptide Institute Inc; Ang II, PD123,319, thapsigargin, ω-conotoxin GVIA, nifedipine, H89, and H7 were obtained from Sigma Biochemicals. Losartan was provided by Merck & Co, Inc.

**Results**

**Effects of Ang II on [Ca²⁺] in Sympathetic Neurons**

As shown in Figure 1, Ang II (200 nmol/L) caused a gradual increase in [Ca²⁺], which persisted for ∼2 minutes before returning to baseline, even in the ongoing presence of Ang II. Interestingly, only a subgroup of neurons (14 of 21) responded to Ang II. The responding neurons typically showed a pattern similar to the previously described slow but transient rise in [Ca²⁺], with an average increase of 54±7% above baseline (Figure 1B). However, 3 of the 14 responding neurons demonstrated clear oscillatory behavior during time...
Figure 1. Differential effects of Ang II on \([\text{Ca}^{2+}]_{i}\) in sympathetic neurons cultured from mouse ARG and CG. A, Superimposed traces were obtained from representative neurons. After a baseline period, Ang II (200 nmol/L) was added to the extracellular solution (black bar). This resulted in a transient increase in the fluorescence ratio (\([\text{Ca}^{2+}]_{i}\)) in one of the neurons (solid line), whereas the other neuron did not respond (broken line). To determine viability, both cells were depolarized with a high potassium solution (stippled bar). B, Effect of Ang II on \([\text{Ca}^{2+}]_{i}\) is summarized for responding (black bar) and nonresponding (stippled bar) neurons. There was no difference in the baseline \([\text{Ca}^{2+}]_{i}\), as shown in the open bars for both groups. C, Effect of potassium depolarization on \([\text{Ca}^{2+}]_{i}\) is summarized for Ang II responding (black bar) and Ang II nonresponding (stippled bar) neurons. Neither the baseline \([\text{Ca}^{2+}]_{i}\) nor the increase in fluorescence ratio after KCl differed between the groups. *P<0.05 Ang II or KCl vs control.

Figure 2. Ang II–induced increase in \([\text{Ca}^{2+}]_{i}\) was mediated by AT1 receptors in sympathetic neurons. Bar graph shows average data of all the neurons tested with losartan and of the responsive neurons tested with PD123,319. The fluorescence ratio represents \([\text{Ca}^{2+}]_{i}\). The AT1 receptor antagonist losartan (2 μmol/L) totally blocked the Ang II response in 11 neurons, whereas 5 of 8 neurons responded to Ang II (200 nmol/L) in the presence of the AT2 receptor antagonist PD123,319 (4 μmol/L). *P<0.05 Ang II vs baseline in presence of PD123,319.

periods up to 10 minutes. There was no difference in the baseline fluorescence ratio between responsive and nonresponsive neurons. To ensure that all neurons were viable, we used a strong nonspecific stimulus, potassium depolarization (KCl, 75 nmol/L), as a positive control (Figure 1C).

To assess whether desensitization contributes to the transient nature of the response to Ang II, a second application of Ang II was performed. Even 20 minutes after removal of Ang II (200 nmol/L) from the extracellular solution, a second administration of the same concentration of Ang II did not evoke a response (increase of 48±6% versus 2±1%, initial versus second application, respectively; n=4). However, in a separate group of neurons, a higher concentration of Ang II (500 nmol/L) did cause a rise in \([\text{Ca}^{2+}]_{i}\), that was less pronounced than the initial Ang II–induced increase in \([\text{Ca}^{2+}]_{i}\), (increase of 60±13% versus 25±10%, initial versus second application, respectively; n=4, P<0.05). Because of this desensitization, we did not perform experiments requiring repetitive administrations of Ang II to the same neuron. When graded concentrations of Ang II were applied to different groups of sympathetic neurons, the increase in \([\text{Ca}^{2+}]_{i}\) was dose-related (Table).

Roles of AT1 and AT2 Receptors in Mediating Response of \([\text{Ca}^{2+}]_{i}\) to Ang II

In the presence of 2 μmol/L losartan, Ang II (200 nmol/L) no longer elicited a \([\text{Ca}^{2+}]_{i}\), increase in any of the neurons tested (Figure 2, n=11). In contrast, \([\text{Ca}^{2+}]_{i}\) rose after Ang II in 5 of 8 neurons after pretreatment with 4 μmol/L PD123,319 (Figure 2). There was no significant difference in the magnitude of the rise in \([\text{Ca}^{2+}]_{i}\), between responsive neurons under control conditions and responsive neurons in the presence of PD123,319 (increase 54±7% versus 41±6%, P=NS).

Role of Ca2+ Influx Versus Ca2+ Release From Intracellular Ca2+ Stores

To investigate the role of Ca2+ influx, we performed experiments in Ca2+-free solution. The removal of extracellular Ca2+ did not affect the baseline \([\text{Ca}^{2+}]_{i}\), presumably because of the short duration (~2 minutes) of exposure to Ca2+-free solution (fluorescence ratio 0.58±0.04 versus 0.54±0.02,
control versus 0 mmol/L Ca\textsuperscript{2+}, respectively; \( P=NS \)). None of the 12 neurons responded to the subsequent administration of Ang II (fluorescence ratio 0.54±0.02 versus 0.55±0.02, control versus Ang II, respectively; \( P=NS \)). After raising [Ca\textsuperscript{2+}] to 2 mmol/L, all the neurons showed a normal increase in [Ca\textsuperscript{2+}], in response to KCl (75 mmol/L), proving their viability (data not shown).

In complementary experiments, the intracellular Ca\textsuperscript{2+} stores were completely depleted by pretreatment with the Ca\textsuperscript{2+}-ATPase inhibitor thapsigargin (500 mmol/L) in Ca\textsuperscript{2+}-free solution. Although caffeine and thapsigargin triggered a transient [Ca\textsuperscript{2+}] increase under control conditions, neither agent caused a change in [Ca\textsuperscript{2+}], after this pretreatment. After depletion of intracellular Ca\textsuperscript{2+} stores, the addition of extracellular Ca\textsuperscript{2+} (2.0 mmol/L) to the extracellular solution led to a rapid increase in [Ca\textsuperscript{2+}], of 25±4% \(( P<0.05, 2.0 \text{ mmol/L } \text{Ca}^{2+} \text{ versus baseline before the addition of extracellular } \text{Ca}^{2+}; \# P<0.05 \text{ Ang II vs baseline before addition of extracellular } \text{Ca}^{2+} \).}

**Role of Voltage-Sensitive Ca\textsuperscript{2+} Channels**

The data described above indicate that the Ang II–induced [Ca\textsuperscript{2+}], increase is mediated by Ca\textsuperscript{2+} influx from the extracellular compartment. This Ca\textsuperscript{2+} influx could be due to the opening of voltage-gated, ligand-gated, or store-operated Ca\textsuperscript{2+} channels. In the presence of 25 mmol/L La\textsuperscript{3+}, the response to Ang II was essentially abolished (Figure 4). Lanthanides are nonspecific channel blockers and may affect several Ca\textsuperscript{2+} influx pathways. Therefore, we investigated the effects of specific Ca\textsuperscript{2+} channel blockers on the Ang II–induced increase in [Ca\textsuperscript{2+}]. Previously published studies showed that N-type Ca\textsuperscript{2+} channels are the predominant pathway for Ca\textsuperscript{2+} influx through voltage-dependent channels in sympathetic neurons.\textsuperscript{15} To determine the contribution of N-type Ca\textsuperscript{2+} channels in Ang II–induced Ca\textsuperscript{2+} influx in ARG and CG sympathetic neurons, the neurons were pretreated with \( \omega \)-conotoxin GVIA, a specific N-type Ca\textsuperscript{2+} channel blocker. Six of 12 neurons responded to Ang II (200 mmol/L) after pretreatment with 2 mmol/L \( \omega \)-conotoxin GVIA (Figure 4), but the response was significantly less than the control response (increase of 18±2% versus 54±7%, \( \omega \)-conotoxin GVIA versus control, respectively; \( P<0.05 \)). Similarly, \( \omega \)-conotoxin GVIA significantly inhibited the KCl-induced increase in [Ca\textsuperscript{2+}], (increase of 66±8% versus 120±12%, \( \omega \)-conotoxin GVIA versus control, respectively). To determine the contributions of L- and P/Q-type Ca\textsuperscript{2+} channels in Ang II–induced Ca\textsuperscript{2+} influx, neurons were pretreated with nifedipine (10 mmol/L) and \( \omega \)-agatoxin IVA (1 mmol/L), which are specific L- and P/Q-type Ca\textsuperscript{2+} channel blockers, respectively. Five of 8 neurons responded to Ang II (200 mmol/L) after pretreatment with nifedipine (Figure 4) with a blunted rise in the fluorescence ratio (increase 32±6% versus 54±7%, nifedipine versus control, respectively; \( P<0.05 \)). The KCl-induced [Ca\textsuperscript{2+}], increase was also significantly inhibited by nifedipine (increase of 88±10% versus 120±12%, nifedipine versus control, respectively; \( P<0.05 \)). In contrast, Ang II–induced and KCl-induced increases in [Ca\textsuperscript{2+}], were not significantly affected by \( \omega \)-agatoxin IVA (for Ang II, increase of 38±6% versus 54±7%, and for KCl, increase of 97±10% versus 120±12%, \( \omega \)-agatoxin-IVA versus control, respectively; \( P=NS \)) (Figure 4).
Role of Protein Kinase Activation in Ang II–Induced Facilitation of Ca2+ Influx

The previously described experiments clearly demonstrate that Ang II triggers Ca2+ influx. To assess whether protein kinase activation is involved in the intracellular signaling pathway, we used an experimental design that assesses Ca2+ influx in response to the addition of extracellular Ca2+ after depletion of intracellular Ca2+ stores. The intracellular Ca2+ stores were completely depleted with thapsigargin in Ca2+-free solution. Subsequently, Ca2+ was added to the extracellular solution in the ongoing presence of thapsigargin, resulting in an increase in [Ca2+], by 21±3% (n=17), taken as control (Figure 5). Pretreatment with Ang II significantly augmented this increase to 54±10% (n=15, Figure 5). Because we could not directly dialyze specific inhibitors into the neurons, we chose membrane-permeable blockers of protein kinase activity at concentrations that preferentially inhibited PKA or PKC. The PKA inhibitor H89 (1 μmol/L) did not affect Ang II–induced enhancement of the rise in [Ca2+], whereas the PKC inhibitor H7 (10 μmol/L) completely blocked the effect (Figure 5).

Discussion

The main findings of the present study are as follows: (1) Ang II triggers a rise in [Ca2+], in a subpopulation of ARG and CG sympathetic neurons; (2) the Ang II effect is mediated by the activation of AT1 receptors; (3) the [Ca2+]i increase is due to Ca2+ influx through voltage-sensitive Ca2+ channels, primarily N-type Ca2+ channels; and (4) activation of PKC is required to mediate these effects of Ang II on ARG and CG sympathetic neurons.

Selectivity of Responsive Neurons

We have recently demonstrated that Ang II triggers a low-amplitude, continuously discharging RSNA in vivo.10 Although hexamethonium eliminated the baseline RSNA, the Ang II–induced change in RSNA persisted in the presence of this ganglionic blocker, suggesting that Ang II affects a subgroup of nerve fibers without requiring synaptic signal transmission. The present study shows that Ang II directly affected isolated ARG and CG sympathetic neurons in vitro, causing a transient rise in [Ca2+]. This effect was limited to approximately two thirds of the neurons. Previously published studies similarly noted that Ang II directly affected neurons in the central or peripheral nervous system.16–25 Whereas some investigators reported responses in all neurons studied,23,24,26–28 others noted effects in 19% to 83% of the neurons examined.16,22,29–32 Our findings and studies from other groups did not specifically address the differences between responsive and nonresponsive neurons. AT1 receptors have been identified in rat superior cervical ganglion (SCG), but the localization of AT1 receptors in individual neurons could not be determined.33 Several studies have shown that sympathetic neurons can be functionally differentiated as phasic and tonic cells on the basis of their electrical response to prolonged electrical stimulation.34–39 Additional studies are needed to determine whether Ang II–responsive neurons correspond to one of these functionally distinct subgroups or whether they exhibit other defining properties.

Differential Responses in Various Autonomic Neurons

The response seen in ARG and CG sympathetic neurons differs from the previously described effects of Ang II on sympathetic neurons from the SCG. Whereas we noted an increase in [Ca2+], Shapiro et al22 did not observe [Ca2+]i changes. In contrast, Ang II suppressed the Ca2+ current by ≈30% in SCG sympathetic neurons.22 Similarly, Ang II inhibited Ca2+ currents in the majority of vagal sensory neurons from nodose ganglia, whereas a small percentage showed an increase in the Ca2+ current.23 Although the AT1 receptor blocker losartan blocked the inhibition of Ca2+ currents in nodose neurons, it did not abolish the facilitation seen in a minority of cells. Experiments with neurons from the brain stem or hypothalamus demonstrated a more consistent effect of Ang II, with a rise in [Ca2+], reported in all studies published.18,19,24,28,32

Ca2+ Influx Versus Ca2+ Release and Ang II–Induced Ca2+ Transients

Changes in [Ca2+]i can be due to Ca2+ influx or Ca2+ release from intracellular stores. Stromberg et al33 and Sumners et
al24 have previously shown that Ang II activates phospholipase C by binding to the AT₁ receptors in the peripheral and central nervous systems. Phospholipase C generates 2 second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (IP₃). Because IP₃ triggers Ca²⁺ release from intracellular Ca²⁺ stores, we first tested whether Ang II elicits a rise in [Ca²⁺], after removal of extracellular Ca²⁺. In contrast to neurons from the area postrema,19 none of the ARG and CG sympathetic neurons studied responded under these conditions. Conversely, sympathetic neurons showed an increase in [Ca²⁺], when Ang II was given after depletion of intracellular stores, thus demonstrating that Ang II triggers Ca²⁺ influx from the extracellular compartment in these neurons. Gebke et al32 reported similar results in neurons from the circumventricular organs. It is not known whether these apparent differences are due to a lack of IP₃-sensitive stores or to differential linking to second-messenger pathways in these cells.

### Voltage-Sensitive Ca²⁺ Channels and Ang II–Induced Ca²⁺ Transients

To further define the pathway activated by Ang II in ARG and CG sympathetic neurons, we initially used a low concentration of lanthanum to block Ca²⁺ channels. Because lanthanum suppressed the response to Ang II, we performed additional experiments with selective Ca²⁺ channel blockers. Sympathetic neurons express several voltage-sensitive Ca²⁺ channels.41-43 Although ω-agatoxin IVA did not significantly alter the response to Ang II, results with ω-conotoxin GVIA and nifedipine indicate that the effect of Ang II primarily requires the activation of N-type Ca²⁺ channels and (to a lesser degree) L-type Ca²⁺ channels in ARG and CG sympathetic neurons. Consistent with 2 studies of Ca²⁺ currents in rat SCG neurons,41,42 we observed that ω-agatoxin IVA did not significantly attenuate KCl- or Ang II–induced increases in [Ca²⁺], suggesting that the P/Q-type Ca²⁺ current does not contribute significantly to the Ca²⁺ influx in these neurons. In nodose neurons, Ang II facilitated Ca²⁺ currents in only a small subgroup of neurons by enhancing Ca²⁺ influx through L-type channels, whereas Ang II suppressed the N-type Ca²⁺ current through an AT₁ receptor–mediated effect in the majority of cells.23 We used an indirect approach to look at changes in Ca²⁺ influx pathways triggered by Ang II. Additional studies are necessary to define whether Ang II directly modulates voltage-sensitive Ca²⁺ channels or whether Ca²⁺ influx is due to depolarization, resulting in activation of Ca²⁺ channels and subsequent Ca²⁺ influx.

### Role of Phosphorylation in Ang II–Induced Ca²⁺ Transients

Our results indicate that phosphorylation through PKC is required to trigger Ca²⁺ influx after the administration of Ang II. Although Bacal and Kunze23 demonstrated that activation of a G protein is required for the inhibition of Ca²⁺ currents in nodose neurons, they did not investigate whether phosphorylation is a necessary step for this effect. Activation of G protein–coupled receptors leads to the dissociation of the βγ G protein–subunit of the heterotrimeric G protein, which may directly interact with the α subunit of the Ca²⁺ channel.44,45 Such a direct effect of the βγ G-protein subunit mediates the effects of Ang II on L-type Ca²⁺ channels in vascular smooth muscle cells.46 Therefore, it is conceivable that the differential effects of Ang II on Ca²⁺ currents described above are due to differences in the intracellular signaling.

### Selectivity of Receptor Type

Ang II elicits its biological actions by binding to specific transmembrane receptors on target cells, thereby activating multiple intracellular transduction pathways. Two major Ang II receptor subtypes have been identified and cloned in mammalian cells:7 AT₁ receptor with its splice variants AT₁₅ and AT₁₆ and the AT₂ receptor. AT₁ and AT₂ receptors have been detected in the brain and other tissues of all mammals studied, including humans.48 Studies with selective blockers have revealed that most of the rapid actions of Ang II in adult animals are mediated by AT₁ receptors.47 In our studies, the Ang II–mediated increase in RSNA in vivo10 and the [Ca²⁺], transient in vitro were both blocked by losartan, demonstrating that the effects were mediated by activation of AT₁ receptors. Similarly, other investigators have reported changes in neuronal function triggered by the activation of AT₁ receptors.18,19,22,24,26,27,31,32

Ang II is known to act on sympathetic ganglia, resulting in modulation of the activity of the sympathetic nervous system. Our data demonstrate that Ang II directly activates ARG and CG sympathetic neurons. The increase in Ca²⁺ influx through voltage-dependent Ca²⁺ channels is indicative of an increase in cellular excitability, which may modulate sympathetic outflow and blood pressure. Further studies in vitro and in vivo are needed to fully characterize the mechanisms activating Ca²⁺ influx through voltage-sensitive channels and to better define the subpopulation of neurons and their role in the control of kidney function, such as renal blood flow, sodium reabsorption, and renin release.

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