Angiotensin II potentiates adrenergic and muscarinic modulation of guinea pig intracardiac neurons

Allison E. Girasole,1 Christopher P. Palmer,1 Samantha L. Corrado,1 E. Marie Southerland,2 Jeffrey L. Ardell,2 and Jean C. Hardwick1
1Department of Biology, Ithaca College, Ithaca New York; and 2Department of Pharmacology, College of Medicine, East Tennessee State University, Johnson City, Tennessee

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CONTROL OF CARDIAC FUNCTION is determined, in part, by the precise coordination of outputs from the sympathetic and parasympathetic branches of the autonomic nervous system. The specific efferent output from each branch is, in turn, controlled by integration of multiple inputs. In addition to inputs from preganglionic neurons, evidence indicates that sensory fibers and local circuit neurons (interneurons) modulate peripheral reflex processing (4, 6, 33). These peripheral autonomic ganglia can function interdependently with central components of the cardiac nervous system for precise beat-to-beat control of cardiac electrical and mechanical functions (4, 6). The intrinsic cardiac plexus, located within the cardiac tissue itself, exemplifies fundamental aspects of this coordinated communication.

The peripheral components of the cardiac nervous system, including the intrinsic cardiac ganglia, express a complex neurochemical profile (19, 33). Previous studies have shown that intracardiac neurons possess receptors for a variety of putative neuromodulators, including muscarinic receptors (7, 24, 38), adrenergic receptors (22, 33), neuropeptides (11, 17), and locally produced chemicals (34). Studies on the direct interactions between the sympathetic and parasympathetic neurons in this plexus are few (26), although the importance of this balance is integral to the normal function of the organism. For example, surgical disruption of the right atrial ganglionated plexus in dogs prevents parasympathetically mediated bradycardia, while maintaining parasympathetically induced suppression of sympathetic inputs modulating chronotropic function (30, 35). This residual response is attributed to sympathetic/parasympathetic neural interactions mediated within the cardiac plexus (30).

Consequently, in the present study, our first objective was to examine the potential for intraganglionic interactions between sympathetic and parasympathetic efferent neurons by determining the effects of both muscarinic and adrenergic neuromodulation on the output of intracardiac neurons. This included characterization of the potential contributions of α and β adrenoceptor subtypes to passive and active membrane properties of intrinsic cardiac neurons.

Previous studies have also demonstrated that neurohumoral interactions can impact functional cardiac control and that these responses are altered with chronic cardiac stress (6). For example, ANG II modulates peripheral autonomic neuronal function (15, 20). ANG II/autonomic interactions remodel with the progression of cardiac disease (1, 13) and, thus, represent novel targets to mitigate adverse disease progression in both cardiac tissues and within the associated cardiac nervous system (37). Stress-induced changes in cardiac tissues and associated neurohumoral regulators can be either detrimental or beneficial. For example, with renin-angiotensin system activation, increased AT1 receptor activity is associated with increased risk of sudden cardiac death (14, 21), while increases in AT2 receptor activation are considered cardioprotective (9, 25, 28). While ANG II is recognized as a putative neuromodulator of peripheral autonomic function, the potential effects of AT1 and AT2 receptors on active and passive membrane properties of efferent neurons of the intrinsic cardiac neurons have not been well characterized. In addition, the potential interactions between ANG II and adrenergic receptor systems...
on intrinsic cardiac neurons have not been considered either, even though both are coactivated during the progression of cardiac disease (6, 13, 30, 36). The potential for ANG II/adrenergic interactions to modulate intracardiac neurons underlie the second objective for the current studies. The data presented here demonstrate that ANG II potentiates both adrenergically and muscarinic mediated activation of intrinsic cardiac neurons, primarily via AT$_2$ receptor-dependent mechanisms.

MATERIALS AND METHODS

**Animals.** Hartley guinea pigs (male, 800–1000 g, Charles River), were euthanized by CO$_2$ inhalation and exsanguination. All procedures were approved by the Institutional Animal Care and Use Committees of Ithaca College and were in accordance with the American Physiological Society’s “Guiding Principles for Research Involving Animals and Human Beings” (3a).

The heart was removed and placed into ice-cold Krebs-Ringer solution (in mM: 121 NaCl, 5.9 KCl, 2.5 CaCl$_2$, 1.2 MgCl$_2$, 1.2 NaH$_2$PO$_4$, 25 NaHCO$_3$, 8 glucose, aerated with 95% O$_2$-5% CO$_2$ for a pH of 7.4). The cardiac plexus, located in the epicardium of the atria, was dissected as previously described (17, 29). The region studied is located primarily in the wall of the left atrium, underlying the area of the coronary sinus, and was exposed by opening the atria and removing the overlying muscle and connective tissue. The isolated plexus was pinned to a Sylgarded-lined 60-mm petri dish and continuously superfused (6–8 ml/min) with 35–37°C Krebs-Ringer solution. Norepinephrine (NE; 10$^{-3}$ M), bethanechol (10$^{-3}$ M), and histamine (10$^{-3}$ M) were applied by local pressure ejection (6–9 psi, Picospritzer; General Valve Corp) through small-tip diameter (5–10 μm) glass micropipettes positioned 50–100 μm from individual neurons. Up to two different drugs could be tested simultaneously by local pressure ejection. For multiple tests of responses in the same cell, the cells were washed with the circulating Krebs solution for several minutes between drug applications. Cells were tested to ensure that all responses had returned to control levels prior to additional testing. ANG II (100 nM) and the AT$_2$ agonist CGP-42112A (Sigma, 100 nM) were applied via the circulating bath solution. Adrenergic agonists (phenylephrine (α$_1$ selective), clonidine (α$_2$ selective), dobutamine (β$_1$ selective), and terbutaline (β$_2$ selective) were applied by either local pressure ejection (10$^{-3}$ M) or by bath perfusion (10 μM). Angiotensin receptor inhibitors telmasartan (AT$_1$ selective, 1 μM Sigma and PD 123319; AT$_2$ selective, 1 μM; Tocris Bioscience) were applied by bath perfusion.

**Electrophysiological methods.** Intracellular voltage recordings from intracardiac neurons were obtained using an AxoClamp 2B amplifier (Axon Instruments) from cells impaled with 2 M KCl-filled microelectrodes (40–80 MΩ). Data were collected, digitized, and analyzed using pClamp 8.2 (Axon Instruments). Individual neurons were used for an experiment when the membrane potential was more negative than −40 mV, and action potentials had an overshoot of at least 20 mV.

Single action potentials were evoked by depolarizing current injection (0.5–0.8 nA, 5 ms), averaged (5 or 6 individual recordings), and analyzed to determine the amplitude and duration (as determined by a return to the resting membrane potential) of the afterhyperpolarizing potential (AHP). Neuronal excitability was monitored by observing the response to a series of long depolarizing current pulses (0.1–0.6 nA, 500 ms). The number of evoked action potentials (AP) vs. stimulus intensity was determined to assess changes in excitability.

Following characterization of the basic electrophysiological properties, induced changes in evoked AP frequency for each cell were assessed immediately following 1–2 s application of either NE or the muscarinic agonist bethanechol. Each was applied by local pressure ejection immediately adjacent (~50–100 μm) to the recorded neuron, and doses were chosen that produce maximal or near-maximal responses. Changes in action potential frequency vs. stimulus intensity were determined to assess relative drug-induced changes in excitability of intrinsic cardiac neurons. ANG II or specific angiotensin receptor agonists were added to the circulating bath solution and neuronal responses to NE, bethanechol, or histamine (applied by local pressure ejection) and were assessed during the superfusion. The bath solution was then switched back to the normal Krebs solution for a total ANG II application time of 2–4 min. Adrenergic agonists were applied either by local pressure ejection or by inclusion in the circulating bath solution. Individual action potentials and neuronal excitability were assessed in the presence and absence of the agonists for either application method.

Inhibition of different ion channels was achieved through the addition of known inhibitors to the bath solution, or omission of specific ions. Tetraethylammonium (TEA; 5 mM) and BaCl$_2$ (1 mM) were added to inhibit potassium channels. CsCl (2 mM) was added to inhibit the hyperpolarization-activated current, Ih, 4-aminopyridine (1 mM) was added to inhibit the A current (IA). Calcium was removed from the Krebs solution, with MgCl$_2$ increased to 4 mM, to prevent influx of extracellular calcium. Solutions were superfused over the tissue, and the neuronal responses to NE and bethanechol were measured before, during, and after application.

**Statistical analysis.** Values are expressed as the means ± SE. Statistical significance was determined by Student’s t-test or ANOVA, with a P value less than 0.05 considered significant. Post hoc analysis was performed using Dunn’s method. Best-fit lines for the frequency curves were generated using either a linear or single exponential relationship, which provided estimates of the $R^2$ values of 0.98 or greater.

RESULTS

**Adrenergic and muscarinic responses.** Whole-mount preparations of the guinea pig cardiac plexus were used to monitor individual neuronal responses to locally applied norepinephrine (NE; 10$^{-3}$M) or the muscarinic agonist, bethanechol (Beth; 10$^{-3}$M). Intracellular voltage recordings from single neurons were used to monitor both passive and active membrane responses. A total of 25 animals were used in this study, and 140 individual cells were sampled.

Application of either NE or bethanechol induced a change in the resting membrane potential in the majority of cells. Approximately 100 cells were tested with NE, and of those, ~70% showed a change in membrane potential or evoked responses, whereas all 70 cells tested responded to bethanechol. The bethanechol response typically consisted of a rapid hyperpolarization (Fig. 1A, 3, mean 6.7 ± 4 mV, 7.6 ± 4 s, n = 14), followed by a slow depolarization (mean 4 ± 2 mV, 39.7 ± 10 s). NE responses showed greater variability in the membrane responses, with ~60% showing a hyperpolarization (Fig. 1A, 2, mean 4.8 ± 2 mV, 16 ± 8 s, n = 12) and 40% showing a depolarization (Fig. 1A, 1, mean 2.8 ± 1 mV, 19 ± 10 s, n = 9). Some neurons showed no measurable change in membrane voltage with NE application, while six cells showed a biphasic response.

Single action potentials were produced by injection of a depolarizing current pulse (0.5–0.8 nA, 5 ms) and averaged. Analysis of the AHP phase of the action potential showed a decrease in AHP duration following application of either NE or bethanechol (Fig. 1B). Prior to application, the average AHP amplitude was 15.8 ± 0.4 mV (n = 37) with a duration of 260 ± 11 ms. Following NE application (n = 14), the AHP amplitude was 15.6 ± 0.9 mV (not significantly different from control).
with a duration of 196 ± 9 ms ($P < 0.001$ by t-test). Similarly, following bethanechol application ($n = 9$), the AHP amplitude was 15.5 ± 1 mV (not significantly different) with a duration of 180 ± 12 ms ($P < 0.001$ by t-test).

Neuronal excitability was assessed by monitoring the number of action potentials produced by a series of depolarizing current steps (0.1–0.6 nA, 500 ms). As shown in Fig. 1C (top), the majority of neurons fired only one or two action potentials at the onset of the depolarizing stimulus. However, following either NE or bethanechol application, the same current pulse produced a significant increase in the number of evoked APs. In addition, bethanechol application consistently resulted in a greater increase in neuronal firing than NE (Fig. 2).

**Ionic mechanisms underlying adrenergic and muscarinic responses.** Several potential ionic mechanisms could underlie the observed responses to NE and bethanechol. Previous studies of NE effects on intracardiac neurons of rats have demonstrated that NE can alter calcium currents (41), while other studies showed that NE can activate a nonspecific cation channel (22). Several muscarinic receptor-mediated changes in ion currents have been described for intracardiac neurons, including inhibition of the M current (32), regulation of the delayed rectifier potassium current (3), inhibition of calcium currents (12), and stimulation of intracellular calcium release (7). To examine whether any of these might contribute to the effects observed in these cells, a variety of inhibitors of different ion channels were added to the circulating Krebs solution. The ability of NE and bethanechol to increase neuronal firing in the presence of 1 mM Ba$^{2+}$ ($n = 4$ cells, Fig. 3B). Additionally, inclusion of either 1 mM 4-aminopyridine (to inhibit the A current, 4 cells) or 2 mM CsCl (to inhibit $I_h$, 4 cells) did not significantly alter the effects of either NE or bethanechol, with all cells still showing an increase in evoked APs following NE or bethanechol application in the presence of the inhibitor (Fig. 3). However, the addition of 5 mM TEA [to inhibit potassium
A total of 31 cells were challenged with at least two different agonists. Of those, seven showed responses to two different agonists. In each case, the cells responded to the $\alpha_1$ agonist and either $\beta_2$ (3 cells), $\beta_1$ (2 cells), or $\alpha_2$ (2 cells).

**ANG II modulation of responses.** Cardiac ganglion preparations were superfused with ANG II to determine its potential to directly modulate intracardiac neuronal activity. ANG II was applied via the addition to the circulating bath solution (100 nM) and was allowed to circulate for several minutes. Single action potentials, neuronal excitability, and responses to NE and bethanechol were assessed in the presence and absence of ANG II. In addition, another known endogenous modulator of intracardiac neuron function, histamine (34), was also examined to determine whether any effects of ANG II on neuronal activity were specific or relatively nonspecific to different potential neuromodulators. ANG II did not alter resting membrane potential or single action potential characteristics (data not shown). The number of evoked action potentials with increased depolarizing current pulses showed a small, but significant, increase with ANG II at the highest stimulus intensity only (Figs. 4B and 5). In the presence of ANG II, application of either NE or bethanechol produced a significantly greater number of action potentials in response to the same stimulus compared with control conditions (Figs. 4 and 5). This response was readily reversible upon removal of ANG II, with excitability responses returning to levels observed prior to ANG II application. In contrast, ANG II did not alter the neuronal response to histamine. Comparison of the effects of ANG II at maximal stimulations for control, NE, bethanechol, and histamine shows a significant increase in evoked action potentials in control, NE, and bethanechol, but not with histamine (Fig. 5).

ANG II works primarily via AT$_1$ and/or AT$_2$ receptors in the regulation of the cardiovascular system (28). While cardiomyocytes have both receptor subtypes, many of the induced functional cardiac responses to ANG II challenge are mediated...
indirectly via modulation of autonomic efferent function (15, 20). To determine which receptors were responsible for the effects observed in this preparation, we used both agonists and antagonists of the specific AT receptor subtypes. Telmasartin, an AT1 receptor antagonist (1 μM), was added to the circulating Krebs solution alone and then in the presence of ANG II. Bethanechol-induced changes in neuronal firing were used to monitor ANG II-mediated effects. Telmasartin did not alter the ANG II-mediated increase in bethanechol-induced neuronal excitability in four different preparations (Fig. 6), Resting membrane potentials: control −44 mV, NE −44 mV, bethanechol −54 mV. B: for each condition (control, NE, and bethanechol), the number of action potentials vs. stimulus intensity was plotted with and without ANG II in the bath. Points represent the means ± SE at each stimulus intensity. The lines represent the best-fit curve to the data. For statistical analysis, a t-test was performed at each individual stimulus intensity, to compare the number of action potentials produced with and without ANG II and *Significant difference, P ≤ 0.05.

DISCUSSION

Integration of information between the sympathetic and parasympathetic nervous system is crucial in the maintenance of cardiac function. The experiments presented here demonstrate that adrenergic neurotransmitters can increase output from intrinsic cardiac neurons via multiple adrenergic receptor subtypes, α-adrenergic being the predominant type. In addition, local stimulation with ANG II can also modulate intra-

Fig. 4. ANG II increases neuronal responses to NE and bethanechol. A: the addition of 100 nM ANG II to the circulating Krebs solution enhanced the increase in the number of action potentials produced with long depolarizing current pulses (0.6 nA, 500 ms) following application (1 s) of either NE or bethanechol. Resting membrane potentials: control −44 mV, NE −44 mV, bethanechol −54 mV. B: for each condition (control, NE, and bethanechol), the number of action potentials vs. stimulus intensity was plotted with and without ANG II in the bath. Points represent the means ± SE at each stimulus intensity. The lines represent the best-fit curve to the data. For statistical analysis, a t-test was performed at each individual stimulus intensity, to compare the number of action potentials produced with and without ANG II and *Significant difference, P ≤ 0.05.
cardiac neuronal function, as well as enhance the increase in excitability induced by both adrenergic and muscarinic agonists. Thus, an increase in sympathetic activity and ANG II production can lead to a concomitant increase in intracardiac neuronal output to the heart. This may represent a feedback loop for parasympathetic blunting of sympathetic responses, or an integration of autonomic outputs within the intrinsic ganglion to modify overall cardiac function.

The guinea pig intracardiac neurons possess both nicotinic and muscarinic receptors (3, 38). The nicotinic receptors are responsible for fast synaptic transmission and lead to suprathreshold excitatory signals. The muscarinic receptors mediate slower responses and several different subtypes of muscarinic receptors are postulated to be expressed by these neurons (3, 7, 12). Thus, muscarinic receptor activation is a likely mechanism for modulation of neuronal output. Application of the muscarinic agonist bethanechol elicited a biphasic change in membrane potential, a shortening of action potential afterhyperpolarization, and an increase in the number of action potentials evoked by long-depolarizing stimuli. This indicates that muscarinic receptor activation can produce an overall increase in neuronal firing in cells where the M current was suppressed by the addition of Ba²⁺. This suggests that mechanisms other than inhibition of I_m contributed to the muscarinic regulation of neuronal excitability. We did find that the muscarinic response was inhibited by extracellular TEA, which would support either modulation of delayed rectifier channels or inhibition of calcium-activated potassium channels, both of which are TEA-sensitive (18). In addition, a muscarinic-induced decrease in calcium currents could also lead to a decrease in calcium-activated potassium channels, which could result in the decrease in AHP duration seen with bethanechol application.

Previous studies of the intrinsic cardiac plexus have shown evidence for adrenergic regulation of intracardiac neurons (5, 26, 38, 41). Norepinephrine directly affected ~70% of guinea pig intrinsic neurons. NE application results primarily in membrane hyperpolarization, although some neurons show a depolarization. NE challenge also resulted in an inhibition of AHP duration, and an increase in evoked action potentials. There was no correlation, however, between the change in membrane potential and a change in evoked action potentials. The results from selective adrenergic agonist challenges indicate a heterogeneous expression of adrenergic receptors within the cardiac plexus, with ~41% of cells showing evidence for α1 receptors, 38% for α2 receptors, 20% for β2 receptors, and 17% for β1 receptors. In addition, a small percentage of neurons showed evidence for expression of multiple adrenergic receptors. The
heterogeneity in adrenergic responses is further seen in the diverse membrane responses, even to a specific agonist challenge. However, by using specific ion channel blockers or removing ions, we were able to demonstrate that the NE-induced increase in evoked action potentials is consistently inhibited by the removal of extracellular calcium ions. Inhibition of potassium channels with Ba$^{2+}$, Cs$, ^+$, 4-AP, or TEA had no effect on the NE-induced increase in evoked action potentials. Thus, although specific receptor expression and membrane responses are heterogeneous, there appears to be a common ionic mechanism underlying the increase in neuronal excitability, with a requirement for extracellular calcium ions to initiate the response.

Increased activation of the sympathetic efferent nervous system is associated with increased production of renin and ANG II. Conversion of ANG I to ANG II occurs not only through plasma angiotensin-converting enzyme activity (ACE), but also via cardiac tissue ACE and chymase produced by mast cells (31). The importance of elevated ANG II levels in cardiac disease is becoming increasingly clear, and studies have shown evidence for negative outcomes associated with increased AT1 receptor activation (14, 21). Conversely, increased activation of AT2 receptors is associated with cardioprotection (9, 10, 25). While cardiomyocytes express AT1 and AT2 receptors, most of the functional changes (electrical and mechanical) induced by ANG II exposure to the heart in vivo are mediated indirectly via induced alterations in autonomic efferent function (15, 20). Thus, we were interested in the potential regulatory effects of ANG II on intracardiac neuron function.

Application of ANG II to guinea pig intrinsic cardiac neurons results in a small, but significant, increase in evoked action potentials at the highest stimulus levels. More pronounced was the synergistic effect of ANG II on the neuronal responses with either adrenergic or muscarinic receptor coactivation. NE application, in the presence of ANG II, resulted in a potentiation of the NE-induced increase in evoked action potentials. Similarly, bethanechol application in the presence of ANG II also produced a significantly greater increase in action potentials at the highest stimulus levels. More profound was the increase in the evoked action potentials in the presence of ANG II.

The results from the characterization of the ANG II receptor subtypes on these cells suggest the expression of AT2 receptors. Application of the AT1 inhibitor telmbsartan did not alter the ANG II responses. The AT2-specific inhibitor PD 123319 did inhibit the ANG II-mediated potentiation of the bethanechol responses, while application of the AT2 agonist CGP 42112A mimicked the ANG II response. These results suggest that modulation of intrinsic cardiac neuronal output by ANG II is mediated via AT2 receptors.

Perspectives and Significance

Integration of sympathetic and parasympathetic efferent neuronal outputs to the heart can occur at the level of the postganglionic neurons (30, 35). The studies presented here show that intracardiac neurons of the guinea pig cardiac plexus can be modulated by both adrenergic signals and hormonal signals, such as ANG II and NE. Both of these neuromodulators can produce an increase in neuronal output, and the combination of NE and ANG II actually produces an even higher evoked output from these neurons.

Given that chronic heart disease can result in increased sympathetic activity, as well as increases in circulating and locally produced ANG II levels (6, 8, 13), these results suggest that the parasympathetic nervous system may compensate for these pathologies, in part, by increasing parasympathetic output. In fact, increased parasympathetic efferent output is correlated with protection against cardiac arrhythmias and sudden cardiac death in the setting of ischemic heart disease (27, 39). Future studies will focus on the impact of these different modulators on the output of intrinsic cardiac neurons with stimulation of fiber inputs. If these modulators are indeed producing functional changes in neuronal excitability, then this should be evident in changes in neuronal firing with synaptic stimulation. Future studies are needed to expand upon the neurochemical/neurohumoral alterations within the cardiac nervous system and the cardiac tissues they innervate during disease progression, with the ultimate goal of developing strategies to mitigate such adverse remodeling, and thereby to help preserve overall cardiac function.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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