Modulation of Angiotensin II Responses in Sympathetic Neurons by Cytosolic Calcium

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Abstract—Both stimulatory and suppressive responses of the sympathetic nervous system to angiotensin II (AII) have been reported in intact animals. To elucidate possible cellular mechanisms, we studied AII-induced changes in cytosolic Ca^{2+} ([Ca^{2+}]i) in primary cultures of rat stellate ganglion neurons. Two different patterns of [Ca^{2+}]i responses to AII were observed: dose-dependent increases in [Ca^{2+}]i in cells with intrinsically low baseline [Ca^{2+}]i (n=64) and dose-dependent suppression of [Ca^{2+}]i in neurons with intrinsically higher baseline [Ca^{2+}]i (n=46). Individual neurons could express both response patterns to AII. In neurons with low basal [Ca^{2+}]i, superfusion with Ca^{2+} ionophore (ionomycin) increased [Ca^{2+}]i and reversed the initial AII-induced stimulatory pattern. L-type Ca^{2+} channel antagonism (nifedipine) in neurons with high baseline [Ca^{2+}]i lowered [Ca^{2+}]i and reversed the initial AII-induced suppressive response. Both stimulatory and suppressive responses were abolished by AT_{1} receptor antagonism (losartan). AII-induced stimulatory responses were blocked by IP_{3} receptor antagonism (2-APB) and by thapsigargin. AII-induced suppression of neuronal [Ca^{2+}]i was blunted when Na-Ca exchange was impaired. We conclude that [Ca^{2+}]i acts as a switch for AII-mediated stimulatory and suppressive responses in individual sympathetic neurons. AT_{1} receptor–mediated neuronal stimulation and suppression may allow local homeostatic adaptation to meet complex systemic needs. (Hypertension. 2003; 41:56-63.)

Key Words: sympathetic nervous system ■ angiotensin II ■ calcium ■ calcium channel blockers ■ norepinephrine ■ inositol

The renin-angiotensin and sympathetic nervous systems are homeostatically interconnected by a complex series of mutually reinforcing and mutually inhibiting interactions. In general, each of these systems tends to reinforce the pressor effects of the other as the two systems act to defend arterial pressure. Sympathetic stimulation is a major controller of juxtaglomerular cell renin release through the actions of catecholamines on juxtaglomerular cell β_{1} receptors.

The angiotensin II (AII) subsequently generated in the bloodstream can exert positive feedback on sympathetic nervous activity by several complex mechanisms, including direct stimulatory actions of AII on central and peripheral sympathetic neuronal activity and catecholamine release.

In addition to its stimulatory effects, AII can also suppress sympathetic neuronal activity in vivo. In animals and humans, complex reflex inhibition occurs when increases in arterial pressure stimulate arterial baroreflexes to cause reflex suppression of sympathetic nervous outflow. A more confusing picture emerges in whole-animal experiments designed to isolate the contributions of the peripheral sympathetic nervous system: AII infusion can facilitate, suppress, or have no effect on norepinephrine release from postganglionic sympathetic neurons. In humans, a similar pattern of sympathetic neural responses has been reported, with some studies showing increased norepinephrine spillover and others showing no response or decreased norepinephrine release in response to AII. In other excitable cell types such as cardiomyocytes or vascular smooth muscle cells, a paradoxical response has also been reported. AII treatment was shown to increase cytosolic calcium ([Ca^{2+}]i) by stimulation of IP_{3}-dependent pathways and was also reported to decrease [Ca^{2+}]i through enhanced calcium efflux.

The current studies were undertaken to examine the effects of AII on neuronal [Ca^{2+}]i and to investigate whether alterations in [Ca^{2+}]i play a role in the apparent “bidirectional” excitatory and suppressive responses to AII seen in sympathetic neurons studied in vivo. Using isolated primary sympathetic neurons from rats, we first related neuronal responses to the dose of AII and to the baseline [Ca^{2+}]i. To confirm that the same neuron is capable of both stimulatory and suppressive responses to AII, cells with spontaneously low [Ca^{2+}]i were exposed to a Ca^{2+} ionophore and cells with spontaneously high [Ca^{2+}]i were exposed to nifedipine. We used losartan to investigate the role of the AT_{1} receptors and confirmed IP_{3} pathway dependency with an IP_{3} receptor blocker and by prior...
calcium depletion. The role of Na-Ca exchange was studied by using Na\(^+\)-free buffers.

**Methods**

**Culture of Rat Stellate Ganglion Neurons**

Details of this method have been described elsewhere. Briefly, adult and newborn (2 to 4 days old) Sprague-Dawley rats were anesthetized with halothane. Thoracotomy-exposed stellate ganglia were removed, rinsed, and minced in Tyrode solution containing (in mmol/L) NaCl 124, KCl 2.6, MgCl\(_2\) 1.2, KH\(_2\)PO\(_4\) 1.2, glucose 5, HEPES 5, NaHCO\(_3\) 3.6, and CaCl\(_2\) 1.5, pH 7.4. Minced neuronal tissues were placed in an enzyme solution containing 0.25% trypsin and 0.1% collagenase and incubated in a 36 °C shaker bath for 30 minutes. The cell suspension was centrifuged at 80 \(\times\) g for 4 minutes. The supernatant was discarded and the dissociated cells were resuspended in growth media containing MEM (Sigma), nerve growth factor-β 100 ng/mL (Sigma), and 10% FBS. Resuspended neurons were plated onto Theranox TM tissue culture coverslips (Lux, Flow Laboratory Inc) precoated with laminin (20 \(\mu\)g/mL). Neurons were plated onto Thermanox TM tissue culture coverslips allowing de-esterification of Fura-2 in the cells and its removal from loaded cells were perfused with Tyrode solution for 20 minutes, allowing de-esterification of Fura-2 in the cells and its removal from loaded cells were perfused with Tyrode solution for 30 minutes in an incubator. The Fura-2-loaded cells were perfused with Tyrode solution for 20 minutes, allowing de-esterification of Fura-2 in the cells and its removal from the extracellular space. Neurons were selected for study after at least 3 days in culture.

**Fura-2 Loading of Sympathetic Neurons**

Fura-2 am (Molecular Probes) was dissolved in DMSO to make a stock solution of 1 mmol/L. Loading solution (6 \(\mu\)mol/L Fura-2) was prepared by vigorously mixing the Fura-2 stock solution with Tyrode solution containing 1% BSA. Neurons in cultures were incubated in the loading solution for 30 minutes in an incubator. The Fura-2-loaded cells were perfused with Tyrode solution for 20 minutes, allowing de-esterification of Fura-2 in the cells and its removal from the extracellular space.

**Measurement of [Ca\(^{2+}\)]\(_i\) Transients**

A coverslip containing Fura-2–loaded cells was mounted in a cell chamber (Biotechics BFC52, Biotechics Inc) that was continuously perfused on the stage of an inverted microscope. A ratio-based microscopic fluorescent spectrophotometer (Photon Technology International Inc) was used to excite the dye at 340/380 nm, alternately at 2-Hz sampling rate. Measurements of 510-nm emissions were recorded as fluorescence ratios (RI/Rmin) from a video screen window encompassing a single neuronal soma. All fluorescence signals were corrected for background by subtracting the average fluorescence intensity in the cell-attached configuration. [Ca\(^{2+}\)]\(_i\) was calculated by the equation [Ca\(^{2+}\)]\(_i\) = Kd · (R - Rmin)/(Rmax - R) · SF/2/Sb2. Kd was determined experimentally by using an in vitro calibration method, with calcium concentrations increased stepwise from 0 to 10 mmol/L. Rmax and Rmin were the fluorescence ratios obtained from the cells exposed to solution containing 1.5 mmol/L CaCl\(_2\), and 15 \(\mu\)mol/L ionomycin, which saturated [Ca\(^{2+}\)]\(_i\), and then to a Ca\(^{2+}\)-free solution containing 10 mmol/L EGTA and 15 \(\mu\)mol/L ionomycin, which would deplete intracellular Ca\(^{2+}\). SF2 and Sb2 were fluorescence intensities at 380 nm with Ca\(^{2+}\) free and Ca\(^{2+}\) saturated solutions, respectively.

**Perfusion System**

Experimental solution (control or drug-containing) flowed continuously through the cell chamber by gravity; the temperature of the perfusate was kept near 36 °C. The volume of fluid in the cell chamber was \(\approx\)0.1 ml; the rate of perfusion was 0.3 to 0.4 mL/min. Perfusate containing drugs could be selected from several reservoirs with multichannel valve without interruption of flow. O$_2$ was continuously bubbled through the perfusion reservoirs.

**Pharmacological Interventions**

Once the baseline [Ca\(^{2+}\)]\(_i\) became stabilized, Ali was administered at different concentrations (1 pmol/L to 1 \(\mu\)mol/L) to neuronal cultures. The role of the AII receptor subtype was determined by administering Ali to the neurons in the presence of the AII type I (AT\(_1\))-receptor blocker losartan and Ali type II (AT\(_2\))-receptor blocker PD-123319. To assess whether the Ali-induced increase in [Ca\(^{2+}\)]\(_i\) was due to intracellular Ca\(^{2+}\) release, neurons with a positive response to Ali were rechallenged by Ali in a Ca\(^{2+}\)-free buffer and after pretreatment with thapsigargin. Furthermore, to determine whether an IP$_3$-dependent mechanism was involved in intracellular Ca\(^{2+}\) release, the effect of Ali was tested in the presence of 2-aminoethoxydiphenyl borate (2-APB, 200 \(\mu\)mol/L, Calbiochem), a specific IP$_3$ receptor blocker. To assess the contribution of the Na-Ca exchange mechanism, Ali experiments were performed under extracellular Na\(^+\)-free conditions. Na\(^+\)-free buffer was prepared by substituting 124 mmol/L Na\(^+\) with equimolar N-methyl-D-glucamine (NMG) or with LiCl (Sigma-Aldrich). Finally, to determine whether altering [Ca\(^{2+}\)]\(_i\) could change neuronal response in the same cells, neurons were exposed to the calcium ionophore ionomycin (3 \(\mu\)mol/L) to raise baseline [Ca\(^{2+}\)]\(_i\) or to the L-type Ca\(^{2+}\) channel blocker nifedipine (1 \(\mu\)mol/L) to lower baseline [Ca\(^{2+}\)]\(_i\). Ali was then administered to these cells after a new baseline [Ca\(^{2+}\)]\(_i\) was obtained.

**Norepinephrine Release Assay**

Primary sympathetic neuronal cultures were prepared as described above. Dissociated stellate ganglion cells pooled from 3 to 5 rats in each preparation were distributed to 3 to 5 collagen-coated culture dishes (Fisher Scientific) to maximize cell density. Culture medium was removed from culture dishes and replaced with a buffer solution containing NaCl 124 mmol/L, HEPES 30 mmol/L, NaHCO\(_3\) 3.6 mmol/L, KCl 4 mmol/L, MgCl\(_2\) 1 mmol/L, CaCl\(_2\) 1.5 mmol/L, glucose 5.6 mmol/L, ascorbic acid 0.2 mmol/L, and 50 mmol/L [H]NE (39 Ci/mmol, Amersham International). After incubation for 50 minutes at 37 °C, the isotope-supplemented medium was removed. The excess [H]NE was washed from the monolayer by 6 consecutive 0.5-mL aliquots of the buffer solution without [H]NE at 37 °C. Release of [H]NE was followed by exposing the monolayer to 0.4 mL buffer for 10 minutes at 37°C in the absence (basal release) and presence of Ali. After each Ali treatment, medium was collected and the monolayers washed with 5 mL buffer solution for 10 minutes. Basal [H]NE release was reestablished before applying new concentrations of Ali. The effect of Ali on [H]NE release was also tested in the presence of losartan (10 \(\mu\)mol/L). Unreleased [H]NE in the monolayer was extracted with perchloric acid (0.4 mol/L) at the end of experiment. The amount of [H]NE released into the medium was expressed as a percentage of the total amount of radioactivity in the cell monolayer at the beginning of the experiment.

**Data Analysis**

The amplitude of Ca\(^{2+}\) transients was measured with Felix software (PTI). Neuronal responses after drug treatment were expressed as mean of [Ca\(^{2+}\)]\(_i\) (mean±SD). The paired Student t test, ANOVA, and Tukey posttest analysis were used for statistical studies. A probability value of \(<0.05\) was considered significant.

**Results**

**Patterns of Neuronal Response to AII**

Primary cultures of neonatal rat sympathetic neurons when exposed to brief pulses (3 minutes) of AII results in acute changes in intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)). Based on morphology of observed Ca\(^{2+}\) tracings, responses were grouped into two categories. In 45% (n=64 of 142 cells) of the neurons tested, Ali exposure resulted in an increase in [Ca\(^{2+}\)]\(_i\) (stimulatory pattern, Figure 1A). This pattern showed an initial phase of rapid transient increase in [Ca\(^{2+}\)]\(_i\) followed by a sustained plateau phase. In 32% (n=46 of 142 cells), Ali caused a paradoxical decrease in [Ca\(^{2+}\)]\(_i\) (suppressive pattern,
Responses when rechallenged (Figure 2B). Treatment with 10 pmol/L AII (data not shown). These results suggested that baseline 

Role of AT1 and AT2 Receptor Subtypes in AII-Induced Responses

To identify AII receptor subtypes involved in mediating changes in [Ca2+]i, specific AT1 and AT2 receptor blockers were used. Neurons that showed stimulatory responses to 10 pmol/L AII were incubated with 10 μmol/L losartan, an AT1 receptor blocker, for 5 minutes. Losartan pretreatment completely abolished stimulatory responses when rechallenged with AII (Figure 2A). In neurons that initially showed suppressive responses to 10 pmol/L AII, pretreatment with 10 μmol/L losartan likewise inhibited these suppressive responses when rechallenged (Figure 2B). Treatment with 1 μmol/L PD-123319, an AT2 receptor blocker, for 5 minutes did not modify the stimulatory or suppressive responses to 10 pmol/L AII (data not shown). These results suggested that both stimulatory and suppressive responses caused by AII were primarily mediated by the activation of AT1 receptors. Involvement of AT2 receptors was not apparent.

Role of Baseline [Ca2+]i in AII-Induced Change in [Ca2+]i

Mean baseline [Ca2+]i for neurons that responded with stimulatory patterns was 78±39 nmol/L (n=64). In neurons that showed suppressive responses, mean baseline [Ca2+]i was 230±48 nmol/L. The difference in mean baseline [Ca2+]i was statistically significant (P<0.01). When data points were arbitrarily segregated into two groups, based on neuronal baseline [Ca2+]i, <200 nmol/L or >200 nmol/L, cells with low baseline [Ca2+]i (<200 nmol/L) predominantly showed increases in [Ca2+]i with AII treatment (n=76, Figure 3). In neurons with high baseline [Ca2+]i (>200 nmol/L), AII largely promoted decreases in [Ca2+]i (n=34, Figure 3). This relation between AII response and baseline [Ca2+]i exists for all AII doses, as shown in the scatterplot and 3-D model (Figure 4). The 3D model derived from 110 aggregated data points correlated baseline [Ca2+]i, AII dose applied, and the [Ca2+]i response to AII (Δ[Ca2+]i). At lower baseline [Ca2+]i, incremental increase in AII dose (1 pmol/L to 1 μmol/L) showed a dose-dependent increase in [Ca2+]i response. On the other hand, at higher baseline [Ca2+]i, increase in AII concentration showed a dose-dependent decrease in [Ca2+]i. These findings suggested that baseline [Ca2+]i in part determined specific patterns of AII-induced responses.

Reversibility of AII Responses Through Control of Baseline [Ca2+]i

To experimentally mimic observational data correlating baseline [Ca2+]i with specific AII responses, baseline [Ca2+]i was manipulated to a desired level. Baseline [Ca2+]i was either raised to a higher level by using a Ca2+ ionophore (ionomycin) or lowered by using an L-type Ca2+ channel blocker (nifedipine). In neurons that initially responded to 100 nmol/L AII with stimulatory patterns (Δ[Ca2+]i=+133±42 nmol/L), baseline [Ca2+]i levels were raised by using 3 μmol/L ionomycin. Ionomycin treatment elevated [Ca2+]i levels from 78±53 nmol/L to 667±150 nmol/L. Rechallenge with the same AII dose (100 nmol/L) after elevation of baseline [Ca2+]i resulted in reversal of responses from stimulatory to suppressive patterns, Δ[Ca2+]i=−438±107 nmol/L (Figure 5A). In neurons that initially responded to 100 nmol/L AII with suppressive patterns, Δ[Ca2+]i=−36±10 nmol/L, baseline [Ca2+]i levels were lowered by using 1 μmol/L nifedipine. Nifedipine treatment modestly lowered [Ca2+]i from 301±12 nmol/L to 244±16 nmol/L. Rechallenge with 100 nmol/L AII after lowering of baseline [Ca2+]i resulted in reversal of responses from suppressive to robust stimulatory patterns, Δ[Ca2+]i=+246±50 nmol/L.
Induction of both stimulatory and suppressive responses in the same neuron through modification of baseline $[\text{Ca}^{2+}]_i$ provided strong evidence that baseline $[\text{Ca}^{2+}]_i$ determined the type of neuronal response to AII.

Involvement of Inositol Trisphosphate Pathway in AII-Induced Stimulatory Responses

To characterize the secondary signals involved in the stimulatory response, the IP$_3$ pathway was investigated. To directly block IP$_3$ receptors, neurons were incubated with 200 μmol/L 2-aminoethoxydiphenyl borate (2-APB) for 5 minutes. 2-APB, by binding noncompetitively to IP$_{3}$ receptors, effectively blocks any IP$_3$-mediated response. Five minutes’ preincubation with 2-APB reduced stimulatory responses by 80% (Figure 6A). 2-APB treatment did not alter suppressive responses to AII (data not shown). To identify the source of Ca stores that resulted in the increase in $[\text{Ca}^{2+}]_i$, neurons that initially showed stimulatory patterns to 100 nmol/L AII ($[\text{Ca}^{2+}]_i = 155\pm 31$ nmol/L) were incubated with 1 μmol/L thapsigargin for 10 minutes (Figure 6B). Restimulation showed minimal increases in $[\text{Ca}^{2+}]_i$ ($\Delta[\text{Ca}^{2+}]_i = +12\pm 7$ nmol/L). Dependence on intracellular $\text{Ca}^{2+}$ stores and inhibition by 2-APB of stimulatory responses indicated that formation of IP$_3$ was necessary for generating stimulatory responses.

Involvement of Na-Ca Exchange in AII-Induced Suppressive Responses

To explore the role of Na-Ca exchange in mediating suppression of $[\text{Ca}^{2+}]_i$, AII exposures were performed under extracellular Na$^+$-free conditions. Na$^+$-free buffers were prepared by equimolar replacement (124 mmol/L) of Na$^+$ with N-methyl d-glucamine (+NMG) or with lithium chloride (+LiCl). Neurons with low baseline $[\text{Ca}^{2+}]_i$ were treated with 3 μmol/L ionomycin to raise $[\text{Ca}^{2+}]_i$. Suppressive responses, $\Delta[\text{Ca}^{2+}]_i = -467\pm 29$ nmol/L, were elicited by AII in these ionomycin-treated cells. Responses were completely inhibited when AII stimulations were performed with NMG buffer (Figure 7A) or LiCl buffer (Figure 7B). Recovery of suppressive responses were obtained when cells were restimulated under regular Na$^+$-containing buffer conditions. Dependence of AII-induced decrease in $[\text{Ca}^{2+}]_i$ on extracellular Na$^+$

(Figure 5B). Induction of both stimulatory and suppressive responses in the same neuron through modification of baseline $[\text{Ca}^{2+}]_i$ provided strong evidence that baseline $[\text{Ca}^{2+}]_i$ determined the type of neuronal response to AII.

Figure 5. Reversibility of AII responses. A, Exposure of low $[\text{Ca}^{2+}]_i$ cells to ionomycin (Iono) were accompanied by reversal of stimulatory effects of 100 nmol/L AII (n=3), demonstrating the ability of a single neuron to exhibit both responses. B, Lowering of $[\text{Ca}^{2+}]_i$ with L-channel blockade showed reversal of initial suppressive responses to AII to a stimulatory response pattern (n=3). *P<0.01, †P<0.05.

Figure 4. Dose-response curve. A, Scatterplot correlating baseline $[\text{Ca}^{2+}]_i$ and response ($\Delta[\text{Ca}^{2+}]_i$) at different AII concentration, at low baseline $[\text{Ca}^{2+}]_i$, AII at all concentrations was stimulatory, whereas at high baseline $[\text{Ca}^{2+}]_i$, AII always exerted a suppressive effect. A neutral effect was seen at $[\text{Ca}^{2+}]_i$ of ~200 and 300 nmol/L. B, Three-dimensional model expands A and shows complex interaction among AII dose, baseline $[\text{Ca}^{2+}]_i$, and subsequent change in $[\text{Ca}^{2+}]_i$. Optimal analysis of modeled data depended on use of multiple doses of AII and presence of widely differing basal $[\text{Ca}^{2+}]_i$ values.
suggested that the Na-Ca exchange plays a role in generation of suppressive responses.

**Norepinephrine Release Profiles**

To assess the role of AII in modulating norepinephrine (NE) release, we compared NE release rates between baseline and post-AII treatment. AII exposure resulted in both stimulatory and inhibitory effects on NE release (Figure 8). In cultures with low baseline NE release (1.7±0.2%/10 minutes), AII induced a significant increase in NE release (n=18). In cultures with an initially high baseline NE release (3.5±0.5%/10 minutes), AII promoted a significant inhibition of NE release (n=10). This bidirectional effect of AII on NE release paralleled the observation seen with AII-induced changes in cytosolic Ca\(^{2+}\).

**Discussion**

The results of this study identify complex postreceptor effects of AII on individual sympathetic neurons and identify the role of cytosolic calcium ([Ca\(^{2+}\)]\(_i\)) as an intrinsic “calcium switch mechanism” that modulates responses to AT\(_1\)-receptor stimulation. In cells with intrinsically low cytosolic calcium ([Ca\(^{2+}\)]\(_i\)), which are presumably quiescent, AII activates IP\(_3\)-mediated release of calcium from the endoplasmic reticulum, thereby activating neurons through acute increases in [Ca\(^{2+}\)]\(_i\). When intraneuronal [Ca\(^{2+}\)]\(_i\) is elevated, AII causes AT\(_1\)-receptor–mediated Na-Ca exchange, which tends to reduce [Ca\(^{2+}\)]\(_i\), presumably passivating the activated neuron. Thus, the neuronal effects of AII (ie, stimulation or suppression) are at least partially dependent on baseline [Ca\(^{2+}\)]\(_i\) and in turn on the cellular mechanisms that affect Ca\(^{2+}\) influx, release, and extrusion. It would appear that each neuron can use its “Ca\(^{2+}\) switch” to modulate its own activity level according to complex needs. After periods of neuronal quiescence, activation of the renin-angiotensin system may enhance and sustain sympathetic activation. During periods of chronic sympathetic activation, however, AII may exert a passivating effect.

The current findings are completely consistent with the well-known tachyphylaxis to infused AII that arises in humans within hours to days, a phenomenon that led to the abandonment of AII as a reliable pressor hormone in humans.16 Our results also verify the apparent divergence in the scientific literature that includes credible descriptions of both stimulatory1,3,17-19 and suppressive effects5,8,20 of AII on the sympathetic nervous system. Finally, the enhancement of AII-dependent neuronal stimulation in the presence of the L-channel blocker nifedipine is consistent with the sympathoexcitation seen during long-term use of this agent in humans21 and may help explain the potential adverse effects observed with calcium antagonists in high-risk patients.22 The finding that both stimulatory and suppressive responses can be elicited in the same neuron argues strongly that the observed physiological effects are not mediated entirely by systemic reflexes. Furthermore, the “bidirectional” effect of AII on sympathetic neurons is clearly not due to activation of different (ie, stimulatory and inhibitory) subclasses of sympathetic neurons within sympathetic ganglia or to the stimulation of different subclasses of AII receptors, because both stimulatory and suppressive effects are blocked by the AT\(_1\)-receptor blocker losartan (Figure 2). Thus, the modulation of AII effects occurs at the postreceptor level and is clearly affected by the cytosolic calcium concentration (Figure 5).

Modulation of neuronal function by AII in vivo could occur as a result of the effects of either locally generated tissue AII or circulating AII. Plasma AII levels are ~5 pmol/L in normal individuals,3 so that the responses to picomolar dosages of AII in the current study are well within the range observed routinely in humans. Quantification of tissue concentrations of AII is considerably more difficult at present, but it has been assumed that locally generated AII modulates neuronal function as well.23 Small changes in plasma AII would be expected to exert significant effects on the sympathetic nervous system because critical AII-dependent sympathetic modulatory effects have been attributed to the actions of AII on circumventricular control centers.
such as the area postrema of the medulla oblongata, which does not have a blood-brain barrier.24 Generation of AII within the central nervous system is also well documented, and the brain synthesizes all components of the renin-angiotensin system within neurons or perineurocytes.23 Whether the observations from this study pertain more to plasma or tissue AII cannot be determined. The current data are also consistent with histochemical findings of high concentrations of AT1 receptors in rat sympathetic ganglion neurons,25,26 including stellate ganglion neurons that provide the primary sympathetic supply to the heart.

At the level of individual neurons, responses to 2-APB (an IP3 receptor blocker) and thapsigargin (which depletes endoplasmic reticular Ca2+ stores) demonstrate that AT1-receptor-mediated increases in [Ca2+]i are due to activation of the phospholipase C-IP3 pathway, with AII-dependent Ca2+ release from the endoplasmic reticulum (ER) (Figure 6). Further support for the idea that AII causes increased [Ca2+]i by releasing ER Ca2+ stores comes from other confirmatory studies in our laboratory demonstrating that removal of Ca2+ from the extracellular buffer only slightly reduces the acute [Ca2+]i responses to AII (data not included). Activation of the phospholipase C-IP3 signaling pathway is a well-known mechanism for AII-mediated cytosolic Ca2+ release from the ER of many types of mammalian cells27–30; the current results extend these findings to include sympathetic neurons.

Suppression of neuronal [Ca2+]i responses to AII appears to be tightly linked to altered Na-Ca exchange. This finding may serve to reconcile existing differences of opinion as to the overall effect of AII on the sympathetic nervous system. Teleologically, AII-induced sympathetic suppression may represent a conservation response that prevents exhaustion of the overall effect of AII on the sympathetic nervous system. Dependent on this suppressive response to AII on Na-Ca exchange is clearly demonstrated by the current experiments that altered the transmembrane sodium gradient in neurons with high baseline [Ca2+]i, using LiCl or NMG, which reduced the Na+ gradient–dependent extrusion of Ca2+ through Na-Ca exchange (Figure 7).

An additional mechanistic link relating cytosolic Ca2+ to modulation of AII-mediated neuronal activation probably occurs at the level of ER IP3 receptors. The degree of binding of IP3 to its ER receptors is tightly regulated by cytosolic Ca2+.31,32 This link may potentially explain the nifedipine responses observed in cells with spontaneously high [Ca2+]i. By decreasing Ca2+ influx, nifedipine may indirectly increase IP3 receptor binding, thereby promoting augmented ER Ca2+ release in response to AT1 receptor stimulation (Figure 5B). There may also be clinical relevance to these findings. t-Type calcium channel antagonists such as nifedipine cause acute and chronic sympathetic activation,33 a phenomenon that may be especially problematic in patients with hypertension, congestive heart failure, and myocardial infarction.22,33–35 Altered baroreflex inhibition could be one mechanism of sympathoinhibition in response to chronic vasodilation, but enhancement of AII-mediated neuronal activation in chronically stimulated sympathetic neurons may be another mechanism underlying chronic sympathoexcitation in high-risk patients with congestive heart failure and acute coronary syndromes.36,37

Whether or not these findings can be fully extrapolated to the intact nervous system remains unclear. The primary advantage of using an in vitro system is the absence of confounding physiological or reflex effects. However, this advantage is also an important drawback because the relative role of cellular and physiological modulating effects cannot be directly compared. Also, potential artifacts of culture preparation cannot be easily eliminated. Cultured sympathetic neurons go through a rigid dissection and isolation procedure that may affect cell membranes and will certainly disrupt cell-cell interactions. The use of nerve growth factor and other constituents of culture media may also influence the results. The fluorescence microscopic technique that was used measures integrated changes in [Ca2+]i over the cell body of individual neurons. Whether these integrated changes represent similar changes in axons or dendrites is also not known. In other cell types, Ca2+ is not uniformly distributed throughout the cytoplasm. Acute Ca2+ transients in many somatic cells occur as dense “sparks” or waves of sparks38,39 rather than uniform increases in [Ca2+]i. Whether comparable [Ca2+]i sparks or waves also occur in sympathetic neurons is not yet known.

These findings of an apparent “calcium switch” mechanism and the associated bidirectional neuronal response to AII open up multiple future research directions. On a cellular level, experiments can be designed to characterize the physiological significance of ±200 nmol/L baseline [Ca2+]i, the critical deflection point for the calcium switch and the
divergent neuronal responses to AII (Figures 3 and 4). The interaction and regulation of IP3 receptors and Na-Ca exchangers should also be examined. Initial reports have suggested that IP3 receptors and Na-Ca exchangers colocalize in the same cellular subdomain.40 This physical proximity may provide further evidence for the counterregulatory function of these two signaling systems. Another area of study that will be of interest is the differential expression of protein isoforms. Several IP3 receptor and Na-Ca exchanger isoforms have been reported; an important question that can be addressed is how changes in isoform expression may affect overall regulation of cytosolic Ca2+. 

Perspectives

Mutually reinforcing stimulatory responses of the sympathetic nervous and renin-angiotensin systems would tend to favor marked vasoconstriction and runaway increases in blood pressure unless a parallel system of negative interactions between the two systems also exists. The current results suggest that both positive and negative interactions between the two systems are controlled at the cellular level and are closely related to the calcium content of the cell. Teleologically, if the sympathetic nervous system (SNS) is chronically activated (neurons with high baseline calcium concentrations), additional stimulation by AII is unnecessary and may even be deleterious. On the other hand, if the SNS has been largely quiescent (cells with low baseline calcium), AII may be useful to extend or amplify the initial stress response. By integrating these whole-animal responses around the calcium metabolism of efferent SNS neurons, a "integrating these whole systems response" may exist that integrates metabolic and neurophysiologic signals without requiring interposed central nervous system inputs or baroreflex modulation. The functional coupling between IP3-mediated Ca release and Ca efflux mediated by the Na-Ca exchanger is evidence of the ability of an individual neuron to integrate different stimuli. Future studies can be directed toward understanding how other cell signals (metabolic, toxic, and so forth) may influence neuronal Ca balance and whether imbalances exist in conditions such as hypertension and heart failure.

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References


